UNIVERSITY OF ALBERTA

CHARACTERIZATION OF CARDIOTONIC EXTRACTS OF SEA ANEMONES FROM THE WEST COAST OF CANADA

by

EDITH IJEOMA CLINE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Pharmaceutical Sciences (Pharmacology)

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Fall 1996

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Votre référence*

Our file *Notre référence*

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-18024-7

Canada

UNIVERSITY OF ALBERTA

LIBRARY RELEASE FORM

NAME OF AUTHOR:	Edith Ijeoma Cline
TITLE OF THESIS:	Characterization of Cardiotonic Extracts of Sea Anemones from the west coast of Canada
DEGREE	DOCTOR OF PHILOSOPHY
YEAR THIS DEGREE GRANTED	1996

Permission is hereby granted to the University of Alberta library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and expect and hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

.....
(Signed)

19A Greenville Lane
Off Wilkonson Rd.
Freetown
Republic of Sierra Leone
West Africa

Date April 20 19 96

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **CHARACTERIZATION OF CARDIOTONIC EXTRACTS OF SEA ANEMONES FROM THE WEST COAST OF CANADA** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY in PHARMACEUTICAL SCIENCES (PHARMACOLOGY).**

.....
Dr. L. I. Wiebe

.....
Dr. J. Samuel

.....
Dr. D. F. Biggs

.....
Dr. J. D. Young

.....
Dr. E. E. Knaus

.....
External Examiner
Dr. Robin J. Marles

Date December 5, 1995

To: My mum and dad

and the late Dr. M. W. Wolowyk

ABSTRACT

Eleven sea anemones species (*Anthopleura elegantissima*, *A. xanthogrammica*, *Corynactis californica*, *Epiactis prolifera*, *Metridium senile*, *Pachycerianthus fimbratus*, *Stomphia didemon*, *Urticina coriacea*, *U. crassicornis*, *U. lofotensis* and *U. piscivora*), collected from the west coast of Canada, were screened for cardiotonic, cytolytic, cytotoxic and antifungal activities.

Extracts from nine species elicited cardiotonic activity on rat atria. While *P. fimbratus* was inactive, *E. prolifera* was cardiotoxic. Eight extracts were hemolytic on erythrocytes of rat, guinea pig and dog; six exhibited cytotoxic activity to KB, L1210 and HEL 299 cell lines. Extracts from all anemone species were devoid of antifungal activity against *Aspergillus niger* PLM 1140 and *Candida albicans* ATCC 14053.

From extracts of *U. piscivora*, three cardiotonic proteins (UpI, UpII and UpIII) were purified using a combination of chromatographic methods.

UpI is a basic protein, (pI > 9.3), of molecular weight ~28 kDa, and N-terminal amino acid sequence of $D^{1}ENEN^{5}LYGPN^{10}ENKAK^{15}AKDLT^{20}AGASY^{25}LTKEA^{30}GCT$ $KL^{35}QAGCT^{40}MYQAY^{45}N$. It elicited potent cardiac stimulatory activity on rat left atria (EC_{50}: $8.1x10^{-9}$ M) comparable to isoproterenol (EC_{50}: $3x10^{-9}$ M). Although different from known anemone proteins, UpI showed some sequence similarity to the bungarotoxins from *Bungarus multicintus*.

UpII and UpIII (pI = 7.2 and 7.6, respectively) appear to be variants of eachother with similar molecular weights of ~19 and 40 kDa in reducing and non-reducing conditions, respectively. Their N-terminal amino acid sequences were $A^{1}TDKW^{5}NDCG$

$S^{10}VTALC^{15}EQKGF^{20}NKATC^{25}$ (UpII), and $D^{1}DDWD^{5}EGCHV^{10}TALLEGQQGR^{20}NK$ AAC^{25} (UpIII). UpII (ED_{50}: $1X10^{-7}$ M) was more potent on rat left atria than UpIII (ED_{50}: $7x10^{-6}$ M).

UpI was hemolytic to erythrocytes of rat, guinea pig, dog, pig and human. Using scanning electron microscopy, it was shown to cause structural damage to the membranes of rat and guinea pig erythrocytes. Hemolysis was inhibited by sphingomyelin. UpII and UpIII were not hemolytic.

Pathophysiological investigations of all proteins revealed only UpI to be toxic, causing hypotension, respiratory and circulatory failure in anesthetized rats, similar to the crude extract from *U. piscivora*. Autopsy revealed morphological damage to the skin, lungs, liver and kidney, but not the heart and brain. All three proteins were devoid of antifungal activity.

While UpI appears to belong to the class of toxins with phospholipase A activity, UpII and UpIII appear to be a new class of cardiotonic proteins.

ACKNOWLEDGMENTS

Very few students find themselves in the unique position I did, while carrying out research for completion of my thesis. I lost my supervisor. However, notwithstanding, I had the help, encouragement and support of a lot of people, for which I will always be grateful.

I wish to express my deep and sincere gratitude to my supervisor, Dr. L. I. Wiebe, for his guidance, advice, moral and financial support throughout the course of my work. It is with his support that this thesis came close to completion. My sincere and thanks appreciation to the Professors who made their labs available to me to carry out some of my research. They are Dr. J. Samuel, Dr. J. D. Young (Chapter 4 and 5) and Dr. D. F. Biggs (Chapter 8), Dr. R. A. Locock (retired) and Dr. E. E. Knaus who made his computers available to me. I am extremely grateful for technical help from Mr. Jack Moore of Alberta Peptide Institute, Dr. Nick Nation, of Animal Lab Services, Dr. Ming Chen of SMRI who taught me how to use the electron microscope, Ms. Carol-Anne McEwen, Dr. Rajan George for his assistance with the biochemical techniques. I wish to thank Dr. Daneshtalab and Ms. Bernadette Oreski of Synphar Laboratories for assistance with biological evaluations of some of my samples.

During my most traumatic moments, my family was always with me, never once giving up on me. To my husband, Ernest, for his encouragement, support and understanding, I say a very big thank you. My sister Milly, and brothers Joe and Robby, together with their spouses, you're the best. I wish to thank all my colleagues and friends particularly Sophie, Nma, Susie and Dr. Arthur Bollo-Kamara for their support.

Financial support for my study was provided by scholarships from University of Alberta/CIDA and Canadian Commonwealth Fellowship and Scholarship Program.

TABLE OF CONTENTS

I. INTRODUCTION 1

1.1 Pharmacologically active marine compounds 2

1.1.1 Cardiovascular activity 2

1.1.2 Cytotoxic and antitumor activity 4

1.1.3 Antimicrobial activity 9

1.1.4 Enzyme inhibitors 11

1.1.5 Other activities of marine organisms 13

1.2 Marine toxins 14

1.2.1 Tetrodotoxin and saxitoxin 14

1.2.2 *Ciguatera* toxins 15

1.2.3 Brevetoxins 18

1.2.4 Palytoxin 18

1.2.5 Conotoxins 19

1.3 The sea anemone 23

1.3.1 Classification of sea anemones 24

1.3.2 General structure of sea anemones 25

1.3.2.1 The tentacles 25

1.3.2.2 The nematocyst 26

1.4 Biological activities of sea anemones 28

1.4.1 Cardiovascularly active polypeptides from sea anemones 29

1.4.2 Cytolytic polypeptides from sea anemones 33

1.4.2.1 Pharmacological effects of sea anemone cytolysins 34

1.4.2.2 Mechanism of action of cytolysins 37

1.4.2.3 Cardiac stimulatory effects of cytolysins 38

1.4.2.4 Cytotoxic and antitumor activity of sea anemone cytolysins 39

1.4.3 Low molecular weight compounds from sea anemones 40

1.5 Biological significance of sea anemone polypeptide toxins 42

Bibliography 44

I. PROPOSED RESEARCH **56**

2.1 Preamble 56

2.2 Objectives 58

Bibliography 59

III. MATERIALS AND METHODS **60**

3.1 Sea anemones 60

3.2 Extraction of sea anemones 62

3.3 *In vitro* experiments 62

3.3.1 Physiological solutions 62

3.3.2 Cardiac stimulatory activity 63

3.3.3 Guinea pig ileal longitudinal smooth muscle (GPILSM) 64

3.3.4 Hemolytic activity 67

3.3.5 Cytotoxic activity 68

3.3.6 Antifungal activity 69

3.4 Separation and purification of cardiotonic proteins 69

3.4.1 Gel filtration chromatography 70

3.4.1.1 Preparation of gel 70

3.4.1.2 Standardization of gel chromatography column 70

3.4.1.3 Separation of crude extracts 71

3.4.2 Ion exchange chromatography 71

3.4.2.1 Choice of ion exchanger 71

3.4.2.2 Separation of active fraction by cation exchange chromatography 72

3.4.3 Reverse phase HPLC 73

3.4.4 Physicochemical characterization of proteins 74

3.4.4.1 SDS-PAGE 74

3.4.4.1.1 Determination of molecular mass of proteins 75

3.4.4.1.2 Confirmation of cardiac stimulatory activity by SDS-PAGE 76

3.4.4.2 Estimation of isoelectric points (pI) of proteins 77
3.4.4.3 Estimation of protein content 77
3.4.4.4 Amino acid analysis 78
3.4.4.5 N-terminal sequence analysis 78
3.4.5 Scanning electron microscopy (SEM) studies 79
3.4.6 Pathophysiological studies 80
3.4.6.1 *In vivo* measurement of blood pressure (BP) and heart rate (HR) 81
3.4.6.2 Histological examinations 81
Bibliography 82

IV. SCREENING OF CRUDE EXTRACTS OF SEA ANEMONES FOR BIOLOGICAL ACTIVITY 84

4.1 Introduction 84

4.2 Results 85

4.2.1 Sea anemones 85
4.2.2 Extraction of sea anemones 88
4.2.3 Cardiac stimulatory activity 89
4.2.4 Cytolytic activity of the extracts 95
4.2.5 Cytotoxic activity of the extracts 99
4.2.6 Antifungal activity of the extracts 100
4.2.7 Protein determination 100

4.3 Discussions and conclusions 102

Bibliography 105

V. ISOLATION AND CHARACTERIZATION OF THE CARDIOTONIC PROTEIN UPI FROM *URTICINA PISCIVORA* 107

5.1 Introduction 107

5.2 Results 109

5.2.1 Sea anemone 109

5.2.2 Taxonomic classification of *U. piscivora* 109

5.2.3 Extraction 109

5.2.4 Purification of the active cardiotonic agent 112

5.2.5 Physicochemical characterization of UpI 116

5.2.5.1 Amino acid analysis of UpI 122

5.2.5.2 N-terminal sequencing of UpI 122

5.2.3 Cardiac stimulatory activity of UpI 124

5.3 Discussion and conclusions 130

Bibliography 135

VI. SEPARATION AND CHARACTERIZATION OF CARDIAC STIMULATORY PROTEIN VARIANTS UPII AND UPIII FROM *URTICINA PISCIVORA*..... 138

6.1 Introduction 138

6.2 Results 140

6.2.1 Sea anemone 140

6.2.2 Separation and purification of cardiotonic variants 140

6.2.3 Physicochemical characterization of protein variants 145

6.2.3.1 Amino acid composition of UpII and UpIII 149

6.2.3.2 N-terminal sequencing of UpII and UpIII 155

6.2.3 Evaluation of cardiac stimulatory and hemolytic activity 156

6.3 Discussion and conclusions 159

Bibliography 162

VII. BIOLOGICAL ACTIVITY OF PROTEINS UPI, UPII AND UPIII FROM *URTICINA PISCIVORA* 164

7.1 Introduction 164

7.2 Results 165

7.2.1 Hemolytic activity of proteins 165
7.2.2 Scanning electron microscope (SEM) studies 168
7.2.3 Cytotoxic activity of proteins 168
7.2.4 Antifungal activity of proteins 171

7.3 Discussion and conclusions 172

Bibliography 175

VIII. PATHOPHYSIOLOGICAL ACTION OF SEA ANEMONE EXTRACTS AND PROTEINS FROM *URTICINA PISCIVORA* 177

8.1 Introduction 177

8.2 Results 177

8.2.1 Isolated smooth muscle experiments 178
8.2.2 Effect on blood pressure (BP) and heart rate (HR) 184
8.2.3 Histologic Examinations 195
8.2.3.1 The lungs 195
8.2.3.2 The skin 200
8.2.3.3 The liver 200
8.2.3.4 The kidney 200

8.3 Discussion and conclusions 207

Bibliography 211

IX. GENERAL DISCUSSION AND CONCLUSIONS 214

9.1 Discussions and conclusions 214

9.2 Future considerations 231

Bibliography 233

LIST OF TABLES

Table — Page No.

1.1 Amino acid sequences of Conotoxins 22

1.2 Pharmacological properties of sea anemone polypeptides and proteins with cardiac stimulatory activity 32

1.3 Polypeptide cytolytic toxins from sea anemones 35

3.1 Sea anemone species used in the research 61

4.1 Approximate yields of extracts of sea anemones 88

4.2 Cardiotonic activity of crude extracts of sea anemones on rat atria 93

4.3 Cytotoxic effect of crude extracts on three cell lines 99

4.4 Protein content (mg/ml) of crude extracts of sea anemones 102

5.1 Amino acid composition of UpI 123

5.2 Partial amino acid sequence of UpI showing the first forty-six residues. 124

5.3 Comparison of N-terminal sequence of UpI with cardiotonic anthopleurins from *Anthopleura elegantissima* and *A. xanthogrammica* 131

5.4 Comparison of N-terminal sequence of UpI with cytolysins from sea anemones 132

5.5 Amino acid sequence homology among UpI and the β-bungarotoxins . 134

6.1 Amino acid composition and isoelectric points of UpII and UpIII 154

6.2 N-terminal amino acid sequences of UpII and UpIII 155

6.3 Comparison of the properties of UpII and UpIII to some other known protein variants 161

7.1 Cytotoxic effect of crude extract of *U. piscivora* and UpI 171

8.1 Summary of postmortem findings from pathophysiological studies 194

9.1 Sensitivity of erythrocytes from three mammalian species to extracts of sea anemones 216

9.2 Comparison of the partial N-terminal sequences of UpI, UpII and UpIII from *U.* piscivora 221

9.3 Comparison of the relative potencies of the cardiac stimulatory proteins UpI, UpII and UpIII from *U. piscivora* 223
9.4 Comparison of characteristics of the cardiotonic proteins UpI, UpII and UpIII from *U. piscivora* 230

LIST OF FIGURES

Figure — Page No.

3.1 Isolated tissue set-up for smooth muscle and atrial tissues 66

4.1 Appearance of sea anemones in a tank of aerated sea water 87

4.2 Typical profile of effect of crude extracts of *Urticina piscivora* on isolated tissues .. 91

4.3 Tracings of the effect of crude extract of *U. piscivora* on rat atrial tissues 92

4.4 Tracings of the effect of crude extract of *Epiactis prolifera* on rat atrial tissues 94

4.5 Relative cytolytic activity of sea anemone extracts to rat erythrocytes 96

4.6 Relative cytolytic activity of sea anemone extracts to guinea-pig erythrocytes 97

4.7 Relative cytolytic activity of sea anemone extracts to dog erythrocytes 98

4.8 Standard curves generated for protein determination of crude extracts of sea anemones 101

5.1 Close up appearance of *U. piscivora* in a tank of aerated sea water 111

5.2 Gel chromatography on Sephadex G-50 M of lyophilized crude extract of *U. piscivora* 114

5.3 Ion exchange purification of active fraction C on SP Sephadex C-25 115

5.4 Analytical reverse phase HPLC of purified UpI 117

5.5 Calibration curve of molecular weight markers from PhastGel SDS-PAGE 118

5.6 Resolution of purified UpI by analytical isoelectric focusing and SDS-PAGE 120

5.7 Calibration curve for pI markers from electrofocusing 121

5.8 Tracing of the effect of UpI (10^{-7} M) on rat left atrium 126

5.9 Dose response curves for UpI and Isoproterenol 127

5.10 Plot of SDS-PAGE of a reverse phase HPLC purified UpI against positive inotropic activity 128

5.11 Schematic representation of the isolation and purification of UpI, II and III 129

6.1 Analytical reverse phase HPLC of fraction 169-192 142

6.2 Analytical reverse phase HPLC of purified UpII 143

6.3 Analytical reverse phase HPLC of purified UpIII 144

6.4 Calibration curve for molecular weight markers from SDS-PAGE 146

6.5 Resolution of purified UpII and UpIII by SDS-PAGE.....148
6.6 Calibration curve for pI markers from electrofocusing.....150
6.7 Resolution of purified UpII and UpIII by analytical isoelectric focusing.....152
6.8 Plot of SDS-PAGE of a reverse phase purified UpII and UpIII.....153
6.9 Tracing of the effects of UpII and UpIII (10^{-7} M) on rat left atria.....157
6.10 Dose response curves for UpII, UpIII and Isoproterenol.....158
7.1 Relative cytolytic activities of UpI measured on erythrocytes of five mammalian species.....166
7.2 Effect of different concentrations of the phospholipid sphingomyelin on hemolytic action of UpI, using different mammalian erythrocytes.....167
7.3 Scanning electron microscopic appearance of membranes derived from distilled water and UpI treated rat and guinea-pig erythrocytes.....170
8.1 Effect of crude extract of sea anemones on the contractile action of guinea-pig longitudinal smooth muscle.....180
8.2 Effect of acetyl choline and its antagonist on the contraction of isolated guinea-pig ileum.....181
8.3 Effect of UpI and its antagonist on the contraction of guinea-pig ileum.....182
8.4 Tracings of the contractile action of 10^{-5} M acetyl choline and UpI on guinea-pig longitudinal smooth muscle.....183
8.5 Effect of crude extract of *Anthopleura xanthogrammica* on arterial blood pressure and heart rate of anesthetized rat.....185
8.6 Effect of crude extract of *Stomphia didemon* on arterial blood pressure and heart rate of anesthetized rat.....186
8.7 Effect of crude extract of *Urticina coriacea* on arterial blood pressure and heart rate of anesthetized rat.....187
8.8 Effect of crude extract of *U. crassicornis* on arterial blood pressure and heart rate of anesthetized rat.....188
8.9 Effect of crude extract of *U. lofotensis* on arterial blood pressure and heart rate of anesthetized rat.....189
8.10 Effect of crude extract of *U. piscivora* on arterial blood pressure and heart

rate of anesthetized rat.....190
8.11 Effect of crude extract of UpI on arterial blood pressure and heart rate of anesthetized rat.....191
8.12 Tracings of the effect of UpI on the arterial blood pressure and heart rate of anesthetized rat.....193
8.13 Appearance of internal organs of rat at autopsy after treatment with UpI.....197
8.14 Histologic manifestations of UpI from *U. piscivora* on rat lung.....199
8.15 Histologic manifestations of UpI on skin of rat.....202
8.16 Histologic manifestations of UpI on rat liver.....204
8.17 Histologic manifestations of UpI on kidney of rat.....206
9.1 Comparison of the maximum responses and dose response of UpI, II and III with Isoproterenol.....225

LIST OF ABBREVIATIONS

A or Ala	Alanine
Af I and II	Toxins from *Anthopleura fuscoviridis*
As I and II	Toxins from *Anemonia sulcata*
B or Asx	Asparagine or aspartic acid
C or Cys	Cysteine
D or Asp	Aspartic acid
E or Glu	Glutamic acid
EDTA	Ethylene diamine tetra-acetic acid
F or Phe	Phenylalanine
FPLC	Fast protein liquid chromatography
G or Gly	Glycine
H or His	Histidine
Hp I-IV	Toxins from *Heteractis paumotensis*
HPLC	High performance liquid chromatography
I or Ile	Isoleucine
ip	Intraperitoneal
iv	Intravenous
K or Lys	Lysine
kg	Kilogram
L	Litres
L or Lys	Leucine
M or Met	Methionine
mg	Miligram
min.	Minute(s)
mm	Milimeter
mM	Milimole
MOPS	3-(N-morpholino) propanesulfuric acid
N or Asn	Asparagine
ng	Nanogram

nM	Nanomole
P or Pro	Proline
Q or Gln	Glutamine
R or Arg	Arginine
S or Ser	Serine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sem	Standard error of the mean
Sh I	Toxin from *Stichodactyla helianthus*
T or Thr	Threonine
TFA	Trifluoroacetic acid
Tris-HCL	Tris[hydroxymethyl]amino methane hydrochloride
µg	Microgram
µl	Microlitre
µm	Micron or micrometer
V or Val	Valine
W or Trp	Tryptophan
Y or Tyr	Tyrosine
Z or Glx	Glutamine or glutamic acid

I. INTRODUCTION

Mankind has known for a long time, at least several thousand years, that living organisms (both marine and terrestrial) contain substances with potent biological activities. Currently, many of the available therapeutic agents stem either directly or indirectly from naturally occurring organic molecules derived from terrestrial plants and/or animals. The abundant flora and fauna inhabiting 70 % of the earth's surface, that portion covered by the oceans, remain relatively unexplored.[1] It was only in the past three decades that significant research activities have suggested that the sea offers an enormous biomedical potential yet to be harnessed by man.

Evidence available to date suggests that the sea offers a rich reserve of novel organic molecules, which, as such, or in structurally modified form(s) may be useful to man either as new drugs to combat diseases or as biomedical, physiological and pharmacological tools in research.[2] As increasing numbers of pathogens become resistant to currently used antibiotics with little ground gained in the war against cancer and with no cure in sight for AIDS, researchers are turning to the sea to find new drugs and antibiotic agents.

Different types of marine organisms are being collected from all around the world, from coral reefs, marshes and the depths of the ocean. Today, the majority of the natural compounds that show promise as anticancer drugs come from the submerged two-thirds of the planet.[3] These bioactive substances have yielded a diverse library of potentially useful compounds with activities that include anticancer, antiviral, antibiotic, anti-inflammatory properties as well as cardiovascular and CNS drugs.[1,3]

The growing awareness of the sea as a potential source of new drugs has stimulated the pharmacological evaluation of pure compounds including some highly potent marine toxins. This is important not only from the point of view of obtaining biomedically useful agents for present or future treatment of diseases, but also for gaining a better knowledge of the pharmacological effects of the toxins. This will in turn result in an effective treatment of human and animal intoxications caused by contact with venoms or ingestion of toxic organisms.

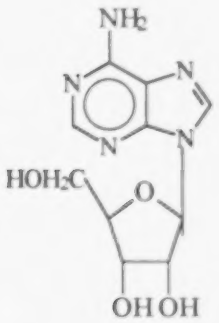
The pharmacological activities of some marine compounds and toxins will be reviewed, illustrating their diverse structural differences.

1.1 Pharmacologically active marine compounds

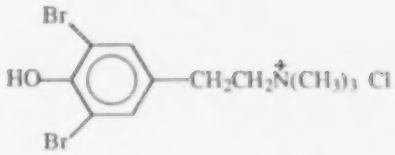
1.1.1 Cardiovascular activity

Extracts of marine animals usually show significant cardiovascular activities on mammalian systems due to the presence of either histamine derivatives or calcium which have been reported to occur in many marine orgarnisms.[4] One of the most notable cardiovascularly active compounds that has been isolated is adenosine (**1**) from the sponge *Dasychalina cyathina*.[5] Adenosine elicits a negative inotropic and chronotropic activity in mammalian heart and this has become the basis for its clinical use for the treatment of supraventricular tachycardia.[6] Its potent vasodilatory action causes an increase in blood flow in hypertensive patients.[7] Autonomium (**2**) is an unusual compound isolated from the sponge *Verongia fistularis* and has been described as a hybrid of adrenaline and acetyl

choline (ACh). It possesses not only α- and β- adrenergic activities but also cholinergic activity similar to ACh.[8]

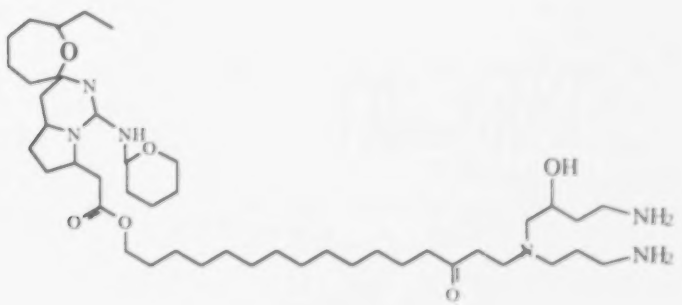


(1)



(2)

Berlinck[9] isolated and characterized alkaloids from the sponge *Crambe crambe*. One of these, crambescidin 816 (**3**), was found to be a potent calcium channel antagonist (IC_{50} 1.5 x 10^{-4} μM) and an inhibitor of ACh-induced contractions in guinea pig ileum.



(3)

1.1.2 Cytotoxic and antitumor activity

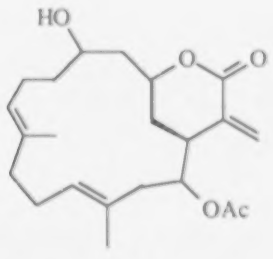
Flam[3] reported that marine organisms today produce the most promising anticancer agents. This may be due to the forces of natural selection to develop elaborate chemical arsenals which are used against predators to avoid being killed. Marine organisms are usually brightly colored and mainly sessile animals which produce powerful toxins which in turn make good cytotoxic agents. The ultimate aim in antitumor therapy is to poison tumor cells more than healthy ones. This has been likened to sessile marine organisms producing toxins which paralyze and kill their prey but not species of their own kind.[10] Today, researchers have developed rapid screening techniques which have revolutionized the search for drugs from natural sources. The US National Cancer Institute (NCI) screens extracts from all over the world on 60 human cancer cell lines, *in vitro*.

A common cytotoxic agent found in marine organisms, particularly soft corals, is the cyclic diterpene, crassin acetate (**4**), isolated from the Caribbean gorgonian *Pseudoplexaura porosa*. It is cytotoxic to mouse fibroblasts, human leukemic and HeLa cells, *in vitro*, at a concentration range of 0.64 - 6.4 mM.[1] Acyclic diterpenes also show cytotoxic activity as was observed for styxenol (**5**) from the sponge *Myrmekioderma styx*. They are also active against P388 murine leukemia and A549 human lung tumor cell lines.[11] The sesterterpene, scalarane (**6**), isolated from the marine sponge *Strepsichordaia*

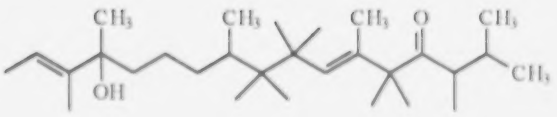
lendenfeldi collected from the Great Barrier Reef of Australia is cytotoxic against P388, A549, HT 29 (human colon carcinoma) and CV1 (monkey kidney fibroblast).[12]

Chemical investigations of the marine tunicate *Eudistoma album* led to the identification of potent cytotoxic agents described as brominated β-carbolines. One such compound is eudistomin E (7), known for its very potent action against KB human buccal carcinoma cells with an LC_{50} less than 1.8 mM.[13]

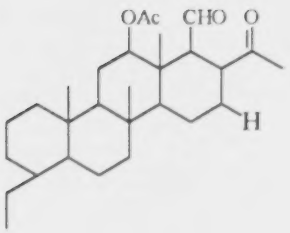
Quite recently, a group of compounds called polyketide peroxides was isolated from the Fijian sponge *Plakortis*. The most striking of them, plakortide E (**8**), showed an interesting *in vitro* cytotoxicity profile on the 60 cell line screen of NCI. It was most potent against melanoma and breast cancer cell lines with LC_{50} values of less than 1 μM. All other derivatives were inactive.[14]



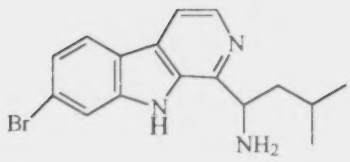
(4)



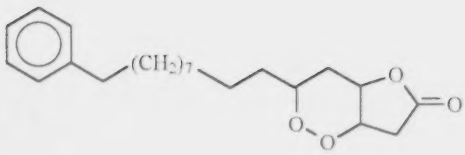
(5)



(6)



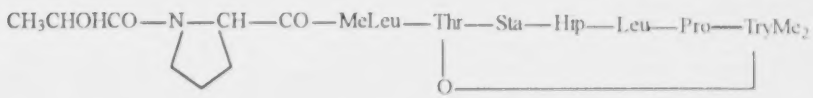
(7)



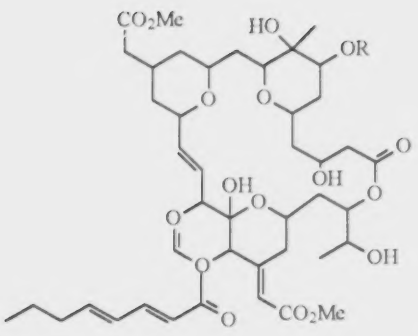
(8)

After more than 25 years of research, marine natural product chemistry is considered to have approached maturity. If the novelty and complexity of the compounds discovered were the only criteria, then success in this area would have been assured. Although a large population of marine compounds have different and interesting pharmacological properties, a commercially successful drug from the sea is still not yet

available. However, five anticancer agents from marine organisms do exist which today are at some stage of clinical or preclinical trials. They are didemnin (**9**) from the tunicate *Tridemnum solidum*,[15] bryostatin (**10**) from the bryozoan *Bugula neritina*,[16] dolasstatin (**11**) from the sea hare *Dolabella auricularia*,[16] halichondrin B (**12**) from the sponges *Lissodendryx* spp.[17] and ecteinascidin (**13**) from the tunicate *Ecteinascidia turbinata*.[18] The oldest of these compounds, didemnin, once thought to be a potent cytotoxin, turned out not to be as efficacious as was thought and because of its toxicity, has been deleted from the clinical trials list by NCI. Bryostatin, on the other hand, is in Phase I and II clinical trials in Europe and Phase I trials in the US. Ecteinascidin is currently being evaluated in preclinical toxicology by NCI and its owners, PharmaMar, while halichondrin is in preclinical trials.[19] The latter shows significant cytotoxicity against all NCI's 60 cell line panel of human tumor cells particularly against B-16 melanoma cells.[17]

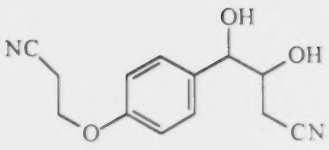


(9)

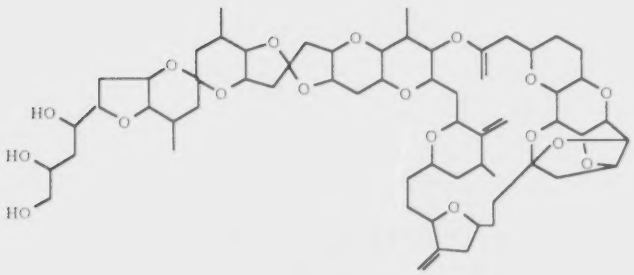


R = Ac or H

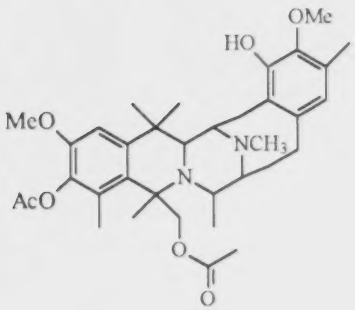
(10)



(11)



(12)

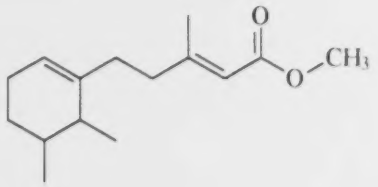


(13)

1.1.3 Antimicrobial activity

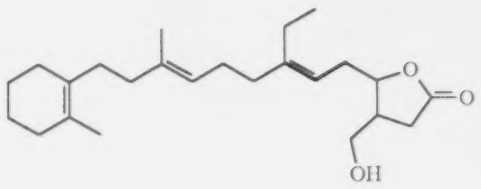
Antibiotic activity is by far the simplest of bioassays carried out today. This has resulted in the evaluation of antibiotic activity of a large number of different classes of marine organisms. Despite such reports, no antibiotic of chemical significance has emerged in the last few decades.[1] This has been reported to be due to several broad spectrum and specifically potent antibiotics of clinical significance from other sources that have emerged in the market recently.

Marine organisms are today recognized as promising targets in the search for new antibiotic drugs and compounds. New compounds possessing antibacterial and antifungal properties from marine sponges, soft corals, bryozoans, tunicates, cnidarians and other invertebrates are still emerging.[20] One such compound is the sesquiterpene sollasin (**14**) from the sponge *Poecillastra sollasi* common in the Bahamas.[21] Sollasin inhibits the growth of two pathogenic fungi *Candida albicans* and *Cryptococcus neoformans*.



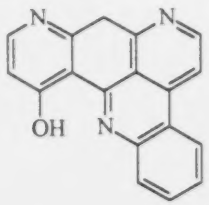
(14)

Whereas sollasin was antifungal, the sesterterpene (**15**) from the sponge *Luffariella* sp. showed strong antibacterial activity against *Escherichia coli* (ATCC 25922), *Bacillus subtilis* and *Micrococcus luteus* (ATCC 9341)[22].



(15)

In their search for antifungal agents of marine origin, McCarthy and his co-workers[23] isolated the potent antifungal and antibacterial agent meridine (**16**) from the sponge *Corticum* sp. It inhibited the growth of *C. albicans*, *C. luteus*, *Trichophyton mentagraphytes* and *Epidermophyton floaosum* at concentrations as low as 0.25 mM. Meridine is active against gram positive but not gram negative bacteria and acts by inhibiting nucleic acid biosynthesis. It is yet to be shown whether marine organisms will yield biomedically or clinically useful compounds with antibiotic activity.



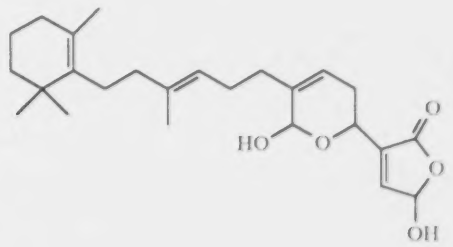
(16)

1.1.4 Enzyme inhibitors

Some of the most painful human ailments involve inflammation, examples of which are arthritis, gout, psoriasis, bee stings and chemically-induced edema. All are characterized by swelling in the affected tissues.

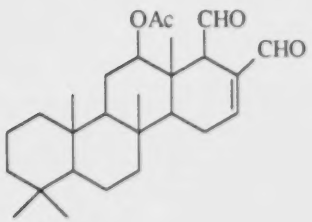
The inflammatory response is mediated by the biosynthesis of eicosanoids from arachidonic acid. Arachidonic acid is produced by hydrolysis of the ester of a membrane phospholipid, a reaction catalyzed by the enzyme PLA_2.[24] Since the release of arachidonic acid provides a substrate for eicosanoid biosynthesis, compounds which inhibit PLA_2 activity have been targeted as potential therapeutic agents in the treatment of inflammation.

The most widely known compound of marine origin with potent PLA_2 inhibitory action is the sesterterpene manoalide (**17**) from the Indo-Pacific sponge *Luffariella variabilis* (IC_{50} 0.04 μM).[25]

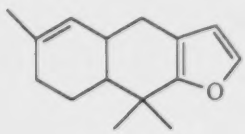


(17)

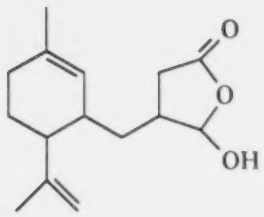
Manoalide inhibits chemically induced edema in mouse ear but not arachidonic acid induced inflammation, an indication that inflammation is inhibited prior to the release of the acid.[26] It also inhibits PLA_2 enzymes from cobra venom[27] and is a potent irreversible inhibitor of human synovial fluid PLA_2 isolated from patients with inflammatory joint diseases.[28] Other known PLA_2 inhibitors include scalaridial (**18**) from the sponge *Cacospongia mollior* (IC_{50} 0.07 μM)[29] and the diterpene glycoside pseudopterosin (**19**) from the Caribbean sea whip *Pseudopterogorgia elisabethae*.[30] The latter inhibits both PLA_2 and lipooxygenase.[24] PLA_2 inhibitors such as γ-hydroxybutenolide (**20**) and gracilin (**21**) have been isolated from the nudibranch *Chromodoris funerea*[31] and the sponge Aplysilla,[32] respectively. A new enzyme inhibitor corallidictyal (**22**), a spirosesquiterpene aldehyde isolated from the sponge *Aka coralliphagum*, showed potent inhibitory action against protein kinase C, an important regulator of cell physiology.[33]



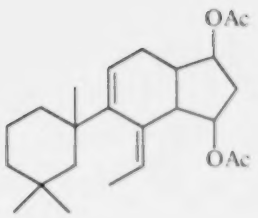
(18)



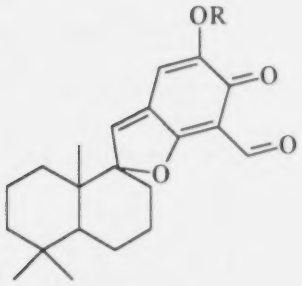
(19)



(20)



(21)



R= H or CH_3

(22)

1.1.5 Other activities of marine organisms

There are several hundred compounds fully characterized from different marine organisms for which only partial or preliminary pharmacological evaluations have been carried out. Some of these compounds have shown hypotensive,[2] antispasmodic,[34] neuromuscular blocking, CNS depressant and antimalarial activities.[35]

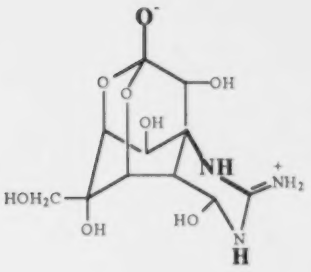
1.2 Marine toxins.

Marine toxins have been of great interest to researchers because of (i), their involvement in human intoxication and (ii), the socioeconomic impacts of these incidents.[36] The chemical structures of marine toxins are usually investigated as this lends some understanding to their mechanisms of action and also provides a model for designing proper countermeasures such as detection and therapeutic protocols. Today, some of the toxins have become useful tools for probing biological or pharmacological phenomena. Some of the most important marine toxins will be reviewed with particular reference to their pharmacological activities.

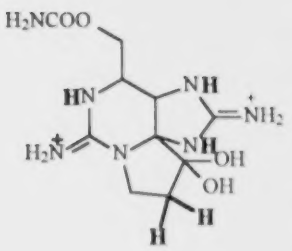
1.2.1 Tetrodotoxin and Saxitoxin

Tetrodotoxin (TTX) (**23**) and saxitoxin (STX) (**24**) are two well known marine toxins, the former for its frequent involvement in fatal food poisoning,[37] and the latter in the fatal food poisoning termed paralytic shell fish poisoning.[1] Both are known for their ability to block sodium channels.

TTX, first thought to be produced by the puffer fish, has since been reported to come from a unicellular marine bacterium which resides within internal organs of the fish.[38] TTX is actually produced by a broad spectrum of marine bacteria.[39] The origin of STX is analogous to TTX.[40]



(23)



(24)

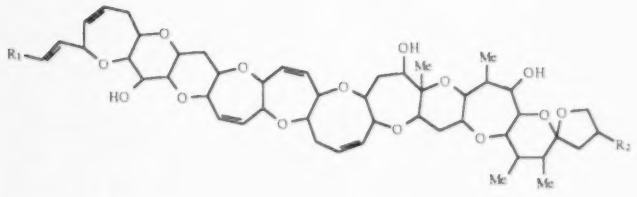
TTX and STX act on the external surfaces of sodium channels to produce a reversible block of the channels. Both have been important tools for studying and understanding the structure of sodium channels and excitable membranes. Both have been used extensively to demonstrate (i), differences in structure of the internal and external regions of the sodium channels, (ii), different binding sites of the channels for monovalent and divalent cations and (iii), differences in pharmacological activities despite their similarities in their physiological actions on nerves, muscles and mammalian cardiac cells.[41]

1.2.2 Ciguatera toxins

Ciguatera is a type of seafood poisoning prevalent in tropical and sub-tropical areas, that is caused by periodic outbursts of dinoflagellates.[1] Poisoning is caused by ingestion of coral reef fishes that become toxic through their diet. Its effect is characterized by neurological, gastrointestinal and cardiovascular syndromes which develop 2 to 24 hours after eating contaminated fish.[36] The two main toxins implicated in

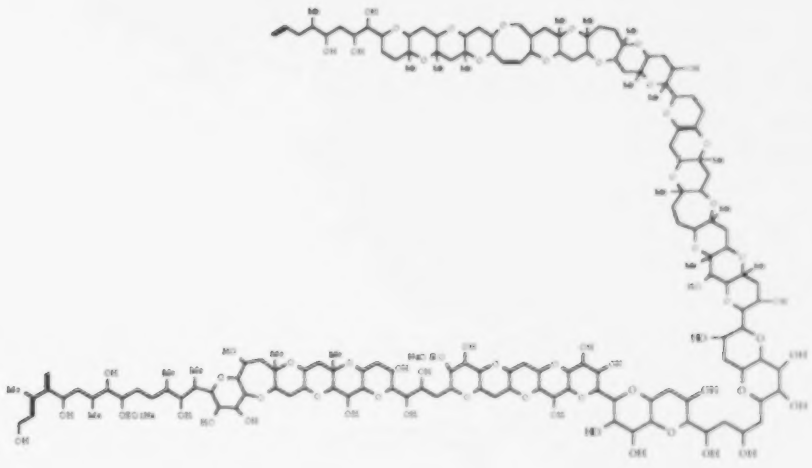
this type of poisoning are ciguatoxin (CTX) (**25**)[42] and maitotoxin (MTX) (**26**),[43] both produced by the dinoflagellate *Gambierdiscus toxicus*.[44,45]

Ciguatoxin is particularly known for reversal of thermal sensation called "dry ice sensation". Other effects include joint pain, miosis, prostration, gastrointestinal disorders such as nausea, vomiting, diarrhea and cardiovascular disturbances such as low blood pressure and bradycardia.[36] In anaesthetized rats and cats, CTX at low doses elicits respiratory stimulation and bradycardia, whereas at higher doses respiratory depression was well marked as bradycardia develop.[46] It elicits potent cardiotonic activity at very low concentrations.[1]

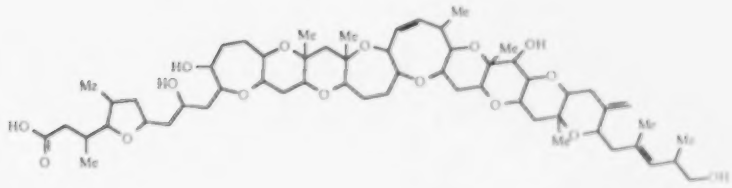


$R_1 = CH(OH)CH_2OH \quad R_2 = OH$

(25)



(26)



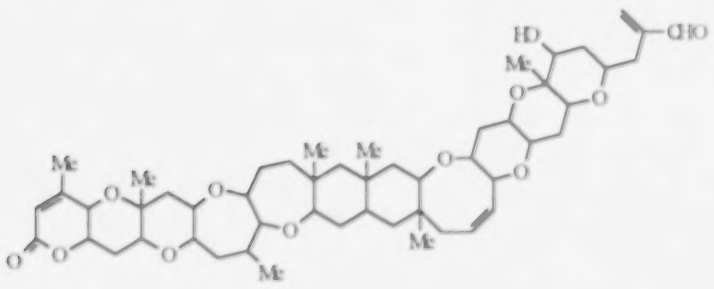
(27)

Maitotoxin has attracted a lot of attention because of its molecular weight (3422 Da), one of the highest for a secondary metabolite.[47] It is extremely potent (LC_{50} 0.17 nM/kg) with a wide range of pharmacological activities that includes muscle contraction, stimulation of hormone and neurotransmitter release and activation of PLA_2.[36]

The other *Ciguatera* toxin isolated from the same dinoflagellate is gambieric acid A (**27**) which is known for its antifungal potency that exceeds that of amphotericin B by a factor of 3,000, making it the most potent antifungal agent known.[36]

1.2.3 Brevetoxins

Brevetoxins are produced by the dinoflagellate *Ptychodiscus brevis* known to form large blooms which cause eye and throat irritation.[48] Brevetoxin B (**28**) causes cardiovascular and respiratory failure,[49] neuromuscular blockade via sodium channels[50] and bronchoconstriction mediated by ACh release.[51,52]

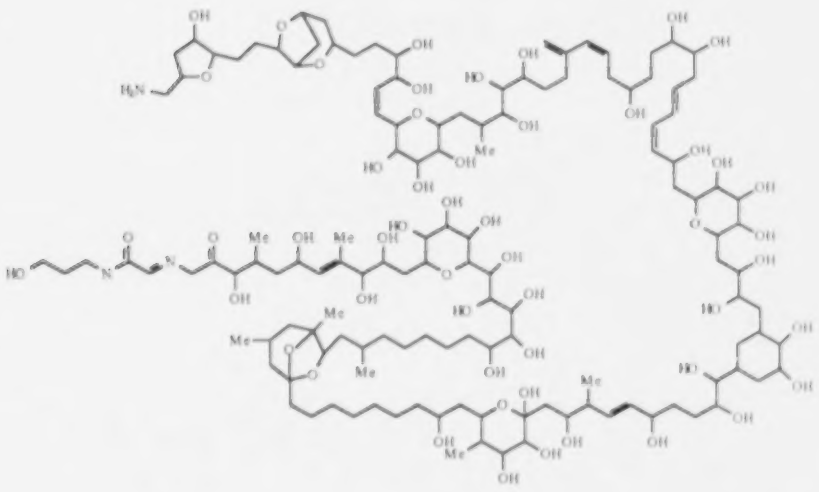


(28)

1.2.4 Palytoxin

Palytoxin (PTX) (**29**), isolated from the marine sea weed *Palythoa* sp.,[53-55] is the most potent marine toxin known. It is a vasoconstrictor that produces total constriction of the coronary artery of isolated guinea pig heart at 1.6×10^{-17} M.[56] Other activities of PTX include induction of smooth muscle contraction,[57] cardiotoxicity in anesthetized

dogs[58] and tumor promoting activity.[36] PTX is a potent hemolysin with a slow course of action and causes a large prelytic potassium loss at LC_{50}'s ranging from 0.03 nM - 5.4 mM.[59] It is also a potent inhibitor of sperm motility at a concentration as low as 10^{-13} M. Because of its unusual structure, several attempts have been made to determine its mechanism of action but to no avail.[1]



(29)

1.2.5 Conotoxins

The deadliness of marine snails of the genus *Conus* spp. (cone snails) was first noted about 300 years ago, but it has only been in the last 7 years that its toxins, designated conotoxins, have been purified and characterized. Cone snails have evolved an unusual biochemical strategy for envenomating their prey[60] and it is reported to involve

the use of small peptides (13-30 amino acids long) with highly potent and specific biological activity.[61] They bind to and inhibit the function of macromolecules in the neuromuscular system.[62]

All *Conus* species are predatory. Kohn[63] established that there were at least 3 feeding types which feed primarily on worms, molluscs and fish respectively. Over 20 human fatalities from *Conus* stings have been reported,[64,65] and *C. geographus* has been responsible for most of these deaths. Symptoms observed in humans after *Conus* stings include numbness at the site of the sting, which spreads to the upper part of the body, blurred vision, impairment of speech, muscle paralysis and ultimately death.[60]

Unlike most polypeptides and proteins of marine origin, which are usually of high molecular weight, *Conus* peptides are low molecular weight peptides that exist primarily as variants in the venom.[66] Today conotoxins are used in hundreds of research laboratories for investigations of both vertebrate and invertebrate nervous systems and as ligands for receptor complexes on the surfaces of cells.[67] Toxicological experiments with rats and guinea pigs have revealed severe respiratory depression and failure accompanied by fluctuations in blood pressure. Death is reportedly due to asphyxiation, caused by paralysis of diaphragm muscle.[68] Three classes of conotoxins are known[69] (Table 1.1) and they are: (i), ω-conotoxins, which inhibit presynaptic calcium channels; (ii), α-conotoxins, inhibitors of ACh receptors and (iii), μ-conotoxins known to inhibit sodium channels on muscle membranes.

ω-Conotoxins were first described[69] as "shaker peptides" because of the neuromuscular effects observed when injected into mice. They do not cause paralysis or death, but rather an uncontrollable shaking syndrome when injected into the CNS. Multiple forms of ω - conotoxins are known and their common feature is the presence of cysteinyl residues which occur as disulfides.[69]

α-Conotoxins, on the other hand, cause paralysis of both mice and fish and they are known to compete with α - bungarotoxins for ACh receptors.[61] Three homologous forms are known, but their potencies vary when injected i.p. in mice.

μ-Conotoxins are a set of homologous, basic, 22 amino acid peptides which block propagation of muscle but not nerve action potential in a similar manner to TTX .[70]

Another peptide from *C. geographus* is a 17 amino acid peptide with a novel biological activity. It induces a sleep-like state when injected into the CNS of mice. The mice remain in this state for 12 - 36 hours and recover without being affected.[69] Several other peptides have been detected in the venom of *Conus* spp., but their pharmacological activities are still unknown. Conotoxins have a great potential in neurobiology and because there are approximately 300 species known, all producing venoms, the cone snails still remain a promising source of neuroactive peptides.

Table 1.1 Amino acid sequences of Conotoxins[62]

Class	Toxin	Sequence
α	GI	Glu-Cys-Cys-Asn-Pro-Ala-Cys-Gly-Arg-His-Tyr-Ser-Cys-NH_2
	GIA	Glu-Cys-Cys-Asn-Pro-Ala-Cys-Gly-Arg-His-Tyr-Ser-Cys-Gly-Lys-NH_2
	GII	Glu-Cys-Cys-His-Pro-Ala-Cys-Gly-Lys-His-Phe-Ser-Cys-NH_2
	MI	Gly-Arg-Cys-Cys-His-Pro-Ala-Cys-Gly-Lys-Asn-Tyr-Ser-Cys-NH_2
μ	GIIIA	Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
	GIIIA	Arg-Asp-Cys-Cys-Thr-Pro-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
	GIIIA	Arg-Asp-Cys-Cys-Thr-Hyp-Pro-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2
	GIIIB	Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Arg-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Met-Arg-Cys-Cys-Ala-NH_2
	GIIIB	Arg-Asp-Cys-Cys-Thr-Pro-Hyp-Arg-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Met-Arg-Cys-Cys-Ala-NH_2
	GIIIB	Arg-Asp-Cys-Cys-Thr-Hyp-Pro-Arg-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Met-Arg-Cys-Cys-Ala-NH_2
	GIIIC	Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Leu-Arg-Cys-Cys-Ala-NH_2
ω-shaker peptide GVIA		Cys-Lys-Ser-Hyp-Gly-Ser-Ser-Cys-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys-Cys-Arg-Ser-Cys-Asn-Hyp-Tyr-Thr-Lys-Arg-Cys-Tyr-NH_2

1.3 The sea anemone

Sea anemones (which display great variability in color) are one of the most abundant of seashore animals. Their coloration is based predominantly upon concentration of carotenoid pigments in combination with certain proteins.[73] There are approximately 1,000 species of sea anemones ranging in size from a few millimeters to half a meter or more in diameter. Most species are as sessile and adults attached to objects of various kinds by their basal disc but are nevertheless able to move about to some extent.[59,72] They feed on molluscs, crustaceans, other invertebrates and fish. Reproduction may be either sexual or asexual.

Geographically, they are world wide in distribution, found in all oceans from the intertidal zone to a depth of about 30,000 feet, with their most elaborate development being found in warmer seas. They can be found on rocky shores, usually preferring slightly sheltered sites even though some species may thrive in regions exposed to the full force of the surf. Some species may be found attached to bedrock, beneath layers of gravel and they have the ability to dig into soft substrata while attached to stone or shells, a phenomenon known as burying. They can tolerate wide ranges of salinity and may occur in lagoons with extreme salinity and temperatures.[59,71] This tolerance for greatly variable environments allows for considerable adaptations among sea anemones, which in turn allows them to survive and reproduce successfully.[73]

1.3.1 Classification of sea anemones

The phylum Cnidaria are simple metazoanns with radial symmetry. These animals are characterized by a simple cylindrical or umbrella-shaped body structure with an internal grastrovascular cavity or coelenteron which opens by a mouth. It is through this opening that food is digested and eliminated. A distinct characteristic of this group of animals is their possession of tentacles. Cnidarians can occur as either sessile polyps or free swimming medusoid forms.[59,71]

The phylum Cnidaria is divided into three classes, the Hydrozoa (hydroids), the Scyphozoa (jellyfish) and the Anthozoans (sea anemones, corals, sea fans, and sea pansies). There are about 9,000 species within this phylum, but only about 70 are capable of causing intoxication in man and other animals.[72] It is estimated that over 30,000 intoxications occur by ingestion of coelenterate marine products, but less than 20 % are properly diagnosed as to the exact etiological agent.[59] Today, the Cnidarians have become one group of marine organisms from which toxicologists seek biologically active principles.

1.3.2 General structure of sea anemones

Sea anemones are soft bodied, highly contractile animals whose shape and movement are governed by a hydrostatic skeleton antagonized by various sheets and blocks of muscles, resulting in the strong characteristic features common to all sea anemones. When covered by water and undisturbed, the body and tentacles are expanded giving them a flower-like appearance.[59]

Their bodies are short hollow cylinders, closed in by a disc at both ends. The upper disc is perforated in the center by a mouth and bears around its edge a fringe of hollow tentacles. Leading inwards from the mouth is a tubular throat or actinopharynx. The cylindrical body is the column and the lower disc the base. The walls of the base, column, disc and throat are uniform in structure and consist of three layers of tissue, the outer cellular ectoderm (epidermis), the inner endoderm (gastrodermis) and a middle layer (mesoglea) consisting of supporting tissues derived from the inner and outer layers.

1.3.2.1 The tentacles

Sea anemones possess tentacles, highly contractile structures which at times may be retracted into the body and covered up by the in-drawing of the upper part of the column. The tentacles may be simple or unbranched structures with smooth surfaces when expanded. In most species, the tentacles develop ectodermal thickenings possessing stinging structures called nematocysts.[74]

1.3.2.2 The nematocyst

Sea anemones possess unique stinging organelles of several types called nematocysts, which play an important role in capturing and paralyzing its prey of crustaceans and small fish.[75] The nematocyst also functions as an organ of defense against predators as well as in intraspecific aggression.[10]

The structure of the nematocyst is used for taxonomic classification of sea anemones. Seventeen different types have been described to date.[76] Although nematocysts are concentrated in the tentacles, they can be found along the entire body of the organism as well as in the gastric filaments.[59] The nematocysts are organelles contained within stinging cells called cnidocytes. They occur as hollow coiled threads which when triggered are everted and used to inject toxins into the body of their prey.[77] Toxins within the nematocysts have been reported to retain their potency within the nematocysts for almost 4 years if stored at low temperatures.[74]

Several researchers have stated that toxins from the nematocyst consist mainly of polypeptides which account for about 95 % of the pharmacologic activity of the animal.[71] Polypeptides and proteins figure predominantly among the various classes of toxins isolated from sea anemone nematocysts. They range in molecular weight from 3,000 to 30,000.[78] Smaller peptides such as the tetrapeptide Antho-RF amide and the pentapeptide Antho-RW amide I and II have more recently been identified from the sea anemone *Anthopleura xanthogrammica*.[79]

The peptide toxins affect conducting system of the mammalian heart,[77,80] as neurotransmitters and neuro-modulators,[81,82] and as membrane active cytolysins.[83,84] Other compounds reported to be present in the nematocyst include amino acids, acid phosphatases, mineral salts, cholinesterases and histamine.[71] Some, or a combination of these compounds, are suggested to be contributing factors to the deleterious effects of some toxins.

1.4 Biological activity of sea anemones

In the last 20 years, researchers have studied the effects of toxins from different members of the cnidarians (sea wasps, sea nettles, jellyfishes) because of the harmful effect of their stings to humans. Interest in the sea anemones arose when it was observed that humans along the Istrian coast of Yugoslavia suffered from stings of the sea anemone *Anemonia sulcata*.[85]

The biologically active compounds of sea anemones comprised mainly of polypeptides and proteins, are divided into four major classes.[83] They are:

(i) Basic polypeptides with molecular weights of 2 - 3 kDa.

(ii) A broader class of polypeptides, molecular weight 4 - 6 kDa, which bind to voltage-gated sodium channels and are either cardiotonic,[86,87] neurotoxic[88] or both.

(iii) Polypeptides with molecular weights ranging from 6 - 10 kDa with proteinase inhibitory activity.[89]

(iv) Membrane active polypeptides called cytolysins with a molecular weight range of 10 - 80 kDa.[90] It has been shown that some polypeptides within this class also possess cardiac stimulatory activity.[91]

1.4.1 Cardiovascular active polypeptides from sea anemones

This group of toxins consists of three classes of polypeptides, two made up of molecules containing 46 - 49 amino acids and the third of 27 - 31 residues. The long polypeptides from the genera *Anthopleura* and *Anemonia* (family Actiniidae) have been classified as Type I and those from Indo-Pacific genera *Heteractis* and *Stichodactyla* (family Stichodactylidae) as Type II.[82,90] Both classes of polypeptides are similar with respect to the locations of disulfide bonds and there is extensive homology (> 60 %) within each class but only about 30 % between the two classes.[77]

The motivation for isolation of polypeptides from *Anthopleura xanthogrammica* and *A. elegantissima* was to the potent cardiac stimulatory action exhibited by their extracts on rat atria.[92] This has led to the isolation of the active principles Anthopleurin A, B and C (AP-A, AP-B and AP-C).[87,93,94] The best characterized of these is AP-A, a potent positive inotropic agent with no significant effects on heart rate or blood pressure *in vivo*.[95] AP-A has the following characteristics:

(i) It elicits a potent dose dependent positive inotropic action on isolated cardiac muscle preparations of rats, guinea pigs, rabbits, cats and dogs both *in vitro* and *in vivo* at concentrations as low as 10^{-9} M.[96,97]

(ii) It is less toxic than other known positive inotropic agents.

(iii) The positive inotropic action of AP-A is not affected when heart tissue preparations are pretreated with receptor blocking agents, indicating that its effect is not mediated by stimulation of these receptors.[92]

(iv) AP-A is reported to be superior to the cardiac glycoside digoxin.[96] The calculated therapeutic index of AP-A is 7.5, compared to 2.5 for digoxin.

(v) Cardiac contractility was more resistant to hypoxia in the presence of AP-A than other cardiac glycosides. It was also maintained at extremely low and high temperatures.[98]

(vi) AP-A elicits positive inotropic activity in calcium-free media as compared to ouabain and INA which are devoid of any action under this condition.

(vii) AP-A (3×10^{-9} - 10^{-8} M) restored contractility to atrial preparations after treatment of heart tissues with calcium channels inhibitors.[98]

Several other polypeptides with cardiac stimulatory activity have been isolated and characterized from different species of sea anemones (Table 1.2). ATX-II is a positive inotrope from *Anemonia sulcata* which shows close similarity to AP-A.[99] A potentially useful therapeutic application of ATX-II is its antiarrythmic activity in dogs. This coupled with its positive inotropic action offers the polypeptide some advantages in the treatment of cardiac conditions.[100] ATX-II is cardiotoxic at high concentrations.

The mechanism underlying the activity of these cardiotonic peptides involves binding to specific site(s) on voltage gated sodium channels of excitable tissues. The major effect of this interaction is a delay of the inactivation process causing the channel to

remain open for longer periods of time. This results in a prolonged action potential and an ultimate positive inotropic action.[101,102] By prolonging the open state of the sodium channel, the toxins enhance sodium influx into the myocardium, leading to a sodium load which in turn affects the release of calcium ions from the sarcoplasmic reticulum (SR) making them available to the contractile elements of the cardiac cell.

Table 1.2 Pharmacological properties of sea anemone polypeptides and proteins with cardiac stimulatory activity[113]

Toxin	LC_{50} (μg/kg)			EC_{50} (nM)	Amino acid
	crab	mice (i.p.)	mice (i.c.)	rat heart	sequence
Type 1					
Af I	100-150	98	-	2	+
Af II	100-150	98	-	2	+
AS II	1.8	100	2.5	15	+
AS I	5.2	19	1.6	2	+
AP-A	11	66	5.3	3	+
AP-B	39	8	0.2	2	+
Type 2					
Hp I	36	145	1.5	3,000	+
Hp II	15	4200	12	5,000	+
Hp III	10	53	2.4	4,000	+
Hp IV	90	40	2	1,300	+
Sh I	0.5-3	15,000	116	8,000	+

EC_{50} median effective concentrations; LC_{50} median lethal dose. The crab species used for assay was the shore crab *Carcinus maenas*.

(-) indicates was not tested; (+) indicates amino acid sequence known.

The nomenclature of the toxins is based on the genus and species (as indicated in list of abbreviations) followed by the Roman numeral to differentiate among multiple toxins from a single species

1.4.2 Cytolytic polypeptides from sea anemones

A number of cytolytic toxins have been found in sea anemones. They represent a unique class of animal toxins with respect to their biochemical characteristics and biological activity. Sea anemone cytolysins are lethal polypeptides which act directly at the membrane level. Some of them have been reported to have cardiac stimulatory activity.[84]

The cytolytic polypeptides of sea anemones (also known as cytolysins) have been classified into three groups based on their molecular weight, isoelectric points and molecular mechanism of action. The three classes according to Macek[83] are: (i), basic toxins with molecular weights of 10 - 20 kDa, comprising more than 27 toxins, (ii), an acidic toxin, metridiolysin (80 kDa), from the sea anemone *Metridium senile*, (iii), phospholipase A_2 (PLA_2) - like toxins (30 kDa) from *Aiptasia pallida*.

Until now, all cytolysins from sea anemones that have been partially or fully characterized have shown characteristics consistent with the first two classes. The cytolysins (Table 1.3) are lethal to invertebrates and vertebrates and they show other biological activities such as cardiac stimulant activity, cardiotoxicity and or cytotoxicity.[83,84]

Although similar in many respects, there are considerable differences in amino acid composition of the cytolysins. They consist mainly of basic amino acids and usually lack cysteine and methionine residues.[84] The exceptions are kentin from *Stoichactis kenti*,[103]

which has one cysteine and one methionine residue, epiactin B from *Epiactis prolifera* which lacks methionine but contains one cysteine residue[104] and the cytolysins from *Condylactis* sp. which have 14 cysteine residues.[84] So far, the complete primary structures of only three sea anemone cytolysins have been elucidated. They are Sh III from *Stoichactis helianthus*,[105] TN - C from *Actinia tenebrosa*[106] and EqT II from *Acitnia equina*.[83] Most of the partially or fully characterized cytolysins show a high degree (as much as 75 %) of structural (amino acid sequence) homology.

1.4.2.1 Pharmacological effects of sea anemone cytolysins

All sea anemone cytolysins presently known, with only one exception, belong to a group of sphingomyelin binding inhibiting proteins with a basic character. The great majority of over 30 toxins were isolated as potent hemolytic agents and additionally some of them have been evaluated for other pharmacological activities.[89,91,107-109]

It has been shown by several authors that the same species of a sea anemone may contain two or more biochemically closely related cytolytic toxins characterized as genetic variants.[90,91,104,110-112]

Using hemolysis assays, cytolysins have been shown to bind to membranes of several mammalian erythrocytes, making them leaky to ions. Species differences in sensitivity to the toxins depend on the particular toxin, but in general rat erythrocytes are the most sensitive, with sensitivity decreasing in the order rat > goat > sheep > horse > human > cat.[113]

Table 1.3 Polypeptide cytolytic toxins from sea anemones[83]

Sea anemone	Toxin	Molecular mass (kDa)	pI	LC_{50} (mg/kg)	Inhibition by phospholipid
Actinia cari	Caritoxin I	19.8	9.45	0.054	SM
	Caritoxin II	19.8	10.0	0.091	SM
Actinia equina	Equinatoxin I	20.0	12.5	0.033	
	Equinatoxin II	19.0	10.5	0.083	SM
Actinia tenebrosa	Tenebrosin A	19.8	>9.4		
	Tenebrosin B	19.8	>9.4		
	Tenebrosin C	19.8	>9.4		
Aiptasia pallida	Fraction III	30.0		0.040	
	Fraction IV	30.0		0.060	
Anthopleura japanicum	Hemolysin I	19.0	>9.0		
	Hemolysin II	19.5	8.0	0.078	
A. xanthogrammica	Lysin	>11.0		9.4	SM
Condylactis gigantea	C.g toxin	18.3	8.9	0.002	SM
Epiactis prolifera	Epiactin A	19.5	6.4		
	Epiactin B	19.5	8.3	0.750	SM
	Epiactin C	19.5	7.6		
Gyrostoma helianthus	G. h hemolysin	10.0		>20.0	SM
Heteractis magnifica	Magnificalysin I	19.0 9.4	0.14		SM
	Magnificalysin II	19.0	0.32		SM

SM: sphingomyelin

Table 1.3 contd.

Sea anemone	Toxin	Molecular mass (kDa)	pI	LC_{50} (mg/kg)	Inhibition by phospholipid
Metridium senile	Metridiolysin	80.0	5.0	0.32	SM/CHOL
Parasicyonis actinostoloides	Parasitoxin	19.0	7.9	0.065	
Pseudactinia varia	Variolysin	19.5	9.8		SM
Radianthus koseirensis	R. k hemolysin	10.0		>20.0	
R. macrodactylus	R. m. toxin	20.0	9.8		SM
Stoichactis helianthus	S. h. toxin	16.0	9.8		SM
Stichodactyla helianthus	Sh I	17.0	8.7		
	Sh II	19.0	9.5		
	S h III	19.2	9.7	0.100	SM
	S h IV	20.0	9.8		
Stoichactis kenti	Kentin	18.0	9.2		SM
Tealia felina	T. f. lysin	-	7.8		
Tealia lofotensis	T. l. lysin	10.0	9.4		SM

SM: sphingomyelin; CHOL: cholesterol

1.4.2.2 Mechanism of action of cytolysins

Bernheimer and Avigad[114] were the first to report the interaction of a sea anemone toxin from *Stoichactis helianthus* with membrane lipids and cell membranes. Sphingomyelin is the main specific receptor molecule although experiments using liposomes of different lipid composition have shown that sphingomyelin is not the sole receptor molecule but the most efficient.[115] Cytolysins have been reported to cause an increase in permeability of cells and model lipid membranes to small ions.[116]

Microscopic examination of lytic events revealed swelling of individual red blood cells before lysis. Zorec[95] suggested a lytic mechanism of colloid-osmotic shock mediated by the formation of pores. Using EqT II from *Anemonia equina*, Belmonte[117] reported the formation of pores with an estimated diameter of 1.1 nm in the erythrocyte membranes. Several other mechanisms of action have been suggested for other cytolysins. For example, the venom component of the aconital nematocyst of *Aiptasia pallida* is reported to be dependent on the presence of calcium ions for its hemolytic action. Separately, individual toxins are slightly hemolytic, but exert a potent hemolytic action when combined.[84] It has been proposed that the toxin first binds to the cell membrane which serves to enhance a phospholipase attack on membrane phospholipid.[118]

Cytolysin III on the other hand binds non-specifically to membrane phospholipids by electrostatic and hydrophobic bonds, followed by the formation of toxin channels or pores across the membranes.[115] The nature of the ion channel formation by cytolysin III was first described by Micheals[119] and later by Varanda and Finkelstein.[116] The former

suggested that three molecules of the toxin aggregated in the lipid bilayer to form the channel while the latter suggested that it was four molecules that brought about this action. Of all the sea anemone cytolysins known, only the hemolytic action of metridiolysin from *Metridium senile* is inhibited by cholesterol.[120] Its interaction with erythrocytes results in the formation of ring-like structures on the membranes similar to what has been observed for bacterial cytolysins.[120]

1.4.2.3 Cardiac stimulatory effect of cytolysins

Besides their primary hemolytic activity, sea anemone cytolysins cause several pharmacological and pathological effects on different tissues and organs. Thompson[121] was the first to show that high molecular weight toxins from sea anemones possess cardiotonic activity. They isolated TN-A, a cardiostimulant protein from *Actinia tenebrosa* with activity at concentrations low as 1.4 nM, and later showed it to be hemolytic. Norton[91] later showed that TN-C from the same sea anemone possessed both activities. Experiments using several pharmacological blocking agents on guinea-pig atria revealed that the cardiac stimulant effect of the tenebrosins was mediated by the release of arachidonic acid from cell membranes by phospholipase A, followed by the synthesis of prostaglandins and leukotrienes. This indirectly brings about a positive inotropic and chronotropic action.[122]

The most widely studied cytolysin with cardiotonic activity is EqT II. It exerts a positive inotropic effect at very low concentrations (1 fM - 0.1 pM). At higher

concentrations a decrease in coronary flow was observed, followed by arrhythmia and negative chronotropism.[123] The mechanism of positive inotropic action of EqT II is said to be similar to TN-C. EqT II also induces pulmonary edema[124] and creates a marked depression in arterial blood pressure and respiration.[125]

1.4.2.4 Cytotoxic and antitumor activity of sea anemone cytolysins

Only few reports describing the cytotoxic and potential antitumor activities of sea anemones exist so far. Extracts of several species have been investigated.[126-129] Tabrah[127] was the first to show that extracts of the sea anemone *Stoichactis sp.* inhibited the growth of Ehrlich ascites tumor by 100 % in mice. This was later followed by Norton and Kashiwagi[128] who isolated the antitumor substance stoichactin from the sea anemone *Stoichactis kenti*. It also inhibited the growth of Ehrlich ascites tumor in mice. Since then the crude aqueous extract of *Anthopleura elegantissima* have been shown to be active against two experimental mouse tumors, P-388 lymphocytic leukemia and Ehrlich ascite tumors *in vivo*.

Of the known cytolysins only EqT II has been thoroughly investigated for both antitumor and cytotoxic actions. Giraldi[108] described the antitumor activity of EqT II and showed that it increased the survival time of mice bearing Ehrlich ascites carcinoma, whereas it had no effect on L1210 leukemia *in vivo*. It elicited a potent *in vitro* action on the two cell lines, with EC_{50} values as low as nM ranges. EqT II has since shown cytotoxicity on V-79-379 cell line[130] and cytostatic activity at 2.6×10^{-6} M. It brings

about morphological alterations to cells as revealed by transmission electron microscopy (TEM).[131]

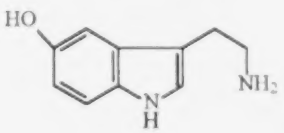
The only other cytolysin evaluated for cytotoxic activity is from the sea anemone *Condylactis aurantiaca* which reduced tumors in melanoma and sarcoma bearing mice.[132] Despite these studies and the promising effects observed *in vitro*, the ability of EqT II and other sea anemone cytolysins in general to inhibit tumor cell growth *in vivo* is only weakly effective.

1.4.3 Low molecular weight compounds from sea anemones

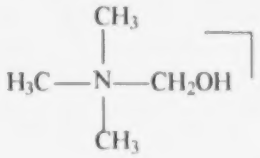
Very few secondary metabolites have been isolated from sea anemones. However it was the symptoms of pain, erythema and edema at the site of nematocyst stings that led to studies involving low molecular weight compounds.[133] One of these compounds, serotonin (**30**), is well known for its pain-producing and histamine-releasing properties.[78] It was first isolated from the sea anemone *Anemonia sulcata* and is a known vasoconstrictor.[133]

Other compounds isolated include tetraethyl ammonium hydroxide (**31**), from *Actinia equina*[133] and histamine (**32**), a known vasodilator, from both *A. sulcata* and *A. equina*.[59] One of the first groups of compounds to be characterized from *Anthopleura* sp. were the naturally occurring phosphonic acid derivatives, 2-methylaminoethyl phosphonic acid (**33a**) and 2-trimethylaminoethyl phosphonic acid (**33b**).[134] Howe and Sheikh[135]

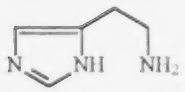
reported the presence of the quaternary ammonium compound, anthopleurine, from *A. elegantissima* which turned out to be an alarm pheromone, which is released by an injury and species causes other anemones of the same species to contract. Zelnik[136] isolated a purine, caissarone (**34**), from the sea anemone *Bunodosoma caissarum*. It induces contraction of longitudinal smooth muscle and rat duodenum and enhances peristaltic contractions at 0.3 mM. The most recent secondary metabolites from a sea anemone are ceramides (**35**) from a hypotensive extract of *Paracondylactis indicus*.[137] Palytoxin (**29**) has also been isolated from *Radianthus macrodactylus*.[138]



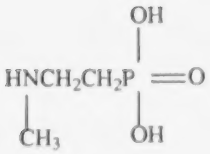
(30)



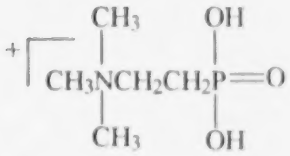
(31)



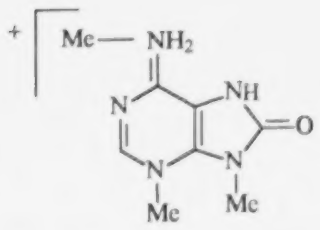
(32)



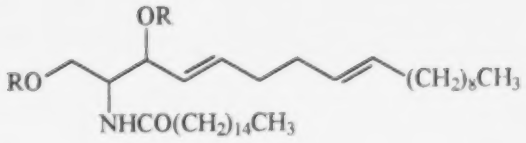
(33A)



(33B)



(34)



R = H or Ac

(35)

1.5 Biological significance of sea anemone polypeptide toxins

The presence of potent toxins in sea anemones is not surprising as they are sedentary and hence require highly effective, quickly acting substances to immobilize and capture their prey and repel potential predators. Two types of toxins are usually found in sea anemones, the lower molecular weight polypeptides with cardiotonic and sometimes neurotoxic activities and the higher molecular weight cytolysins.[113] It has been shown by some researchers that sea anemones which possess cardiotonic polypeptides usually lack cytolysins and *vice versa*.[84,113] The only exceptions are *Parascicyonis actinostoloides*[139,140] and *Stoichactis helianthus*,[90,141] in which both types of toxins have been detected.

The specific biological role of these toxins have been difficult to assess for a long time, mainly because of fragmentary information on their toxicology, together with the fact that their actual localization within the organism is unknown. The only anthozoan polypeptides known to have come from the nematocyst are the hemolysins (for which no

sequence information is available) from the sea anemone *Aiptasia pallida*.[142] If sea anemone toxins occur predominately within the tentacles then it can be assumed that their roles will be due to a predator-prey relationship. All of the toxins known to date have been shown to be lethal to crustaceans and or small fish upon which they feed.[110,140] Thus, in a situation where a sea anemone possesses both low and high molecular weight toxins, it can be concluded that such species would be most effective against a broad spectrum of potential prey or predators. Some cytolysins are known to function as aids to food digestion instead of serving as toxins.[143]

Bibliography

1. Kaul, P. N. and Daftari, P. (1986) Marine pharmacology: Bioactive molecules from the sea. *Ann. Rev. Pharmacol. Toxicol.* **26**, 117-142.

2. Kaul, P. N. (1982) Biomedical potential of the sea. *Pure and Appl. Chem.* **54**, 1963-1972.

3. Flam, F. (1994) Chemical prospectors scour the seas for promising drugs. *Science* **266**, 1324-1325

4. Hollenbeak, K. H., Schmitz, F. J., and Kaul, P. N. (1976) *Food and Drugs from the Sea Proceedings.* (Webber, H. H. and Ruggieri, G. D. eds.) pp. 282-287, Marine Technological Society, Washington D. C.

5. Kaul, P. N. (1976) A cardioactive compound isolated from the sponge *Dasychalina cyathina. Food and Drugs from the Sea.* (Webber, H. H. and Ruggieri, G. D. eds.) pp. 288-296, Marine Technological Society, Washington D. C.

6. Evans, D. B., Weishaar, R. E. and Kaplan, H. R. (1982) Strategy for the discovery and development of a positive inotropic agent. *Pharmacol. Ther.* **16**, 303-330.

7. Davis, L. P., Jamieson, D. D., Baird-Lambert, J. A. and Kazlauskar, R. (1984) Halogenated pyrrolopyrimidine analogues of adenosine from marine organisms. Pharmacological activities and potent inhibitor of adenosine kinase. *Biochem. Pharmacol.* **33**, 347-355.

8. Hollenbeak, K. H., Schimitz, F. J., Kaul, P. N. and Kulkarni, S. K. (1978) A dual adrenergic compound from the sponge *Verongia fistularis. Drugs and Food from the Sea-Myth or Reality.* (Kaul, P. N. and Sinderman, C. J. eds.) pp. 81-87, Oklahoma University Press, Norman, OK.

9. Berlinck, R. G. S., Braekman, J. C., Daloze, D., Bruno, I., Riccio, R., Ferri, S., Spampinato, S. and Speroni, E. (1993) Polycyclic guanidine alkaloids from the marine sponge *Crambe crambe* and Ca^{2+} channel blocker activity of crambescidin 816. *J. Nat. Prod.* **56**, 1007-1015.

10. Brace, R. C. (1990) Aggression in a sea anemone: a model of early non-self recognition. *Endeavour, New Series* **14**, 159-162.

11. Sennett, S. H., Pomponi, S. A. and Wright, A. E. (1992) Diterpene metabolites from two chemotypes of the marine sponge *Myrmeikioderma styx. J. Nat. Prod.* **55**, 1421-1429.

12. Bowden, B. F., Coll, J. C., Li, H., Cambie, R. C., Kernan, M. R. and Berquist, P. R. (1992) New cytotoxic scalarane sesterterpenes from the dictyoceratid sponge *Strepsichordaia lendenfeldi*. *J. Nat. Prod.* **55**, 1234-1240.

13. Adesanya, S. A., Chbani, M., Pais, M., and Debitus, C. (1992) Brominated β - carbolines from the marine tunicate *Eudistoma album*. *J. Nat. Prod.* **55**, 525-527.

14. Varoglu, M., Peters, B. M. and Crews, P. (1995) The structures and cytotoxic properties of polyketide peroxides from a *Plakortis* sponge. *J. Nat. Prod.* **58**, 27-36.

15. Reinhart, K. L., Gloer, J. B., Cook, J. C., Mizsak, S. A. and Scahill, T. A. (1981) Structures of didemnins, antiviral and cytotoxic depsipeptides from a Caribbean tunicate. *J. Am. Chem. Soc.* **103**, 1857-1859.

16. Petit, G. R., Herald, C. L., Dubek, D. L., Herald, D. L., Arnold, E. and Clardy, J. (1982) Isolation and structure of bryostatin I. *J. Am. Chem. Soc.* **104**, 6846-6848.

17. Hirata, Y. and Uemura, D. (1986) Halichaondrins - antitumor polyether manoalide from a marine sponge. *Pure and Appl. Chem.* **58**, 701-710.

18. Wright, E. A., Forteo, D. A., Gunawardana, G. P., Gunasekera, S. P., Koelu, F. E. and McConnel, O. J. (1990) Antitumor tetrahydroisoquinoline alkaloids from the colonial ascidian *Ecteinascidia turbinata*. *J. Org. Chem.* **55**, 4508-4512.

19. Newman, D. (1995) Anticancer marine natural products at NCI under different stages of clinical trials (Personal communications).

20. Antonio, J. and Molinski, T. F. (1993) Screening of marine invertebrates for the presence of ergosterol-sensitive antifungal compounds. *J. Nat. Prod.* **56**, 54-61.

21. Killday, K. B., Longley, R., McCarthy, P. J., Pomponi, S. A., Wright, A. E., Neale, R. F. and Sills, M. A. (1993) Sesquiterpene-derived metabolites from the deep water marine sponge *Poecillastra sollasi*. *J. Nat. Prod.* **56**, 500-507.

22. Konig, G. M., Wright, A. D. and Sticher, O. (1992) Four new antibacterial sesterterpenes from a marine sponge of the genus *Luffariella*. *J. Nat. Prod.* **55**, 174-178.

23. McCarthy, P. J., Pitts, T. P., Gunawardana, G. P., Kelly-Borges, M. and Pomponi, S. A. (1992) Antifungal activity of meridine, a natural product from the marine sponge *Corticum* sp. *J. Nat. Prod.* **55**, 1664-1668.

24. Potts, B. C. M., Faulkner, D. J. and Jacobs, R. S. (1992) Phospholipase A_2 inhibitors from marine organisms. *J. Nat. Prod.* **55**, 1701-1717.

25. de Silva, E. D. and Scheuer, P. J. (1980) Manoalide, an antibiotic sesterterpenoid from the marine sponge *Luffariella variabilis* (Polejaeff). *Tetrahedron Lett.* **21**, 1611-1614.

26. de Freitas, J. C., Blaukemeier, L. A. and Jacobs, R. S. (1984) *In vitro* inactivation of the neurotoxic action of β-bungarotoxin of the marine natural product manoalide. *Experientia.* **40**, 864-865.

27. Glaser, K. B. and Jacobs, R. S. (1986) Molecular pharmacology of manoalide: Inactivation of bee venom phospholipase A_2. *Biochem. Pharmacol.* **35**, 449-453.

28. Jacobson, P. B., Marshall, L. A., Sung, R. S. and Jacob, R. S. (1990) Inactivation of human synovial fluid phospholipase A_2 by marine natural product manoalide. *Biochem. Pharmacol.* **39**, 1557-1564.

29. de Carvalho, M. S. and Jacobs, R. S. (1991) Two step inactivation of bee venom phospholipase A_2 by scalaridial. *Biochem. Pharmacol.* **42**, 1621-1626.

30. Roussis, V., Wu, Z., Fenical, W., Strobel, S. A., van Duyne, G. D. and Clardy, J. (1986) New antiinflammatory pseudopterosins from the marine octoral *Pseudopterogorgia elisabethae*. *J. Org. Chem.* **55**, 4916-4922.

31. Carte, B., Kernan, M. R., Barrabee, E. B., Faulkner, D. J., Matsumoto, G. M. and Clardy, J. (1986) Metabolites of the nudibranch *Chromodoris funerea* and the singlet oxygen oxidation products of furodysin and furodysinin. *J. Org. Chem.* **51**, 3528-3532.

32. Mayol, T., Piccialli, V. and Sica, D. (1985) Gracilin A, a unique nor-diterpene metabolite from the marine sponge *Spongionella gracilis*. *Tetrahedron Lett.* **26**, 1357-1360.

33. Chan, J. A., Freyer, A. J., Carte, B. D., Hemling, M. E., Hofmann, G. A., Mattern, M. R., Mentzer, M. A. and Westley, J. W. (1994) Protein kinase C inhibitors: novel spirosesquiterpene aldehydes from a marine sponge *Aka (Spihnoodictyon) coralliphagum*. *J. Nat. Prod.* **57**, 1543-1548.

34. Sjoblom, T., Bohlin, L. and Christopheren, C. (1983) Studies of Swedish marine organisms. 2. Muscle relaxant alkaloids from marine bryozoan *Flusta foliacea*. *Acta Pharm. Suec.* **20**, 415-419.

35. Jacobs, M. (1995) Biotechnology and drug firms farm bioproducts from the sea. *Genetic Engineering News* **15**, 1-19.

36. Yasumoto, T. and Murata, M. (1993) Marine toxins. *Chem. Rev.* **33**, 1897-1909.

37. Koa, C. Y. and Levinson, S. R. (1986) *Tetrodotoxin, Saxitoxin and the Molecular Biology of the Sodium Channel.* The New York Academy of Sciences. New York

38. Yasumoto, T., Yotsu, M., Endo, A., Murata, M. and Naoki, H. (1989) Interspecies distribution and biogenetic origin of tetrodotoxin and its derivatives. *Pure Appl. Chem.* **61**, 505-508.

39. Noguchi, T., Jeon, J. K., Arakawa, O., Sugita, H., Deguchi, H., Shida, Y. and Hashimoto, K. (1986) Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio sp.* isolated from the intestines of the xanthid crab *Atergatis floridus*. *J. Biochem.* **99**, 311-314.

40. Kodoma, M., Ogata, T. and Sata, S. (1988) Bacterial production of saxitoxin. *Agric. Biol. Chem.* **52**, 1075-1077.

41. Strichartz, G. and Castle, N. (1990) Pharmacology of marine toxins: Effects on membrane channels. *Marine toxins: Origin, structure, and molecular pharmacology.* (Hall, S. and Strichartz, G. eds.) pp. 3-20, American Chemical Society, Washington DC.

42. Scheuer, P. J., Takahashi, W., Tautsumi, J. and Yoshida, T. (1967) Ciguatoxin: Isolation and chemical nature. *Science* **155**, 1267-1268.

43. Murata, M., Naoki, H., Iwashita, T., Matsunaga, S., Sasaki, M., Yokoyama, A. and Yasumoto, T. (1993) Structure of maitotoxin. *J. Am. Chem. Soc.* **115**, 2060-2062.

44. Adachi, R. and Fukuyo, T. (1979) The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* (gen. et. nov) collected in a ciguatera endemic sea. *Bull. Jpn. Soc. Sc. Fish.* **45**, 67-71.

45. Yasumoto, T., Nakajima, L., Oshima, Y., and Bagnis, R. (1979) Toxic dinoflagellate blooms. (Taylor, D. L. and Seliegner, H. eds.) pp. 65 Elseveir, North Holland.

46. Legrand, A. M., Galonnier, M. and Bagnis, R. (1982) Studies on the mode of action of ciguateric toxins. *Toxicon* **20**, 311-315.

47. Yokoyama, A., Murata, M., Oshima, Y., Iwashita, T. and Yasumoto, T. (1988) Some chemical properties of maitotoxin, a putative calcium channel agonist isolated from a marine dinoflagellate. *J. Biochem.* **104**, 184-187.

48. Baden, D. G. (1983) Marine food-borne dinoflagellate toxins. *Int. Rev. Cytol.* **82**, 99-150.

49. Borison, H. L., Ellis, S. and McCarthy, L. E. (1980) Central respiratory and circulatory effects of *Gymnodinium breve* toxin in anaesthetized cats. *Br. J. Pharmacol.* **70**, 249-256.

50. Shinnick-Gallagher, P. (1980) Possible mechanism of action of *Gymnodinium breve* toxin at the mammalian neuromuscular junction. *Br. J. Pharmacol.* **69**, 373-378.

51. Baden, D. G., Mende, T. J., Bikhazi, G. and Leung, I. (1982) Bronchoconstriction caused by Florida red tide toxins. *Toxicon* **20**, 929-932.

52. Baden, D. G. and Mande, T. J. (1981) Toxicity of two toxins from the Florida red tide marine dinoflagellate *Ptyohodiscus brevis*. *Toxicon* **20**, 457-461.

53. Armstrong, R. W., Bean, J. M., Cheon, S. H., Christ, W. J., Fujioka, H., Ham, W. H., Hawkins, L. D., Jin, H., Kang, S. H., Kishi, Y., Martinelli, M. J., MeWhorter, W., Mizumo, M., Nakata, M., Stutz, A. E., Talamas, F. X., Taniguchi, M., Tino, J. A., Ueda, K., Uenishi, J., White, J. B. and Yonaga, M. (1989) Total synthesis of palytoxin carboxylic acid and palytoxin amide. *J. Am. Chem. Soc.* **111**, 7530-7533.

54. Moore, R. E. and Bartoloini, G. J. (1981) Structure of palytoxin. *J. Am. Chem. Soc.* **103**, 2491-2494.

55. Uemaura, D., Ueda, K. and Hirata, Y. (1981) Further studies on palytoxin. II. Structure of palytoxin. *Tetrahedron Lett.* **22**, 2781-2784.

56. Habermann, E. (1989) Palytoxin acts through Na^+, K^+ - ATPase. *Toxicon* **27**, 1171-1187.

57. Ozaki, H., Tomono, J., Nagase, H. and Urakawa, N. (1983) The mechanism of contractile action of palytoxin on vascular smooth muscle of guinea-pig aorta. *Jpn. J. Pharmacol.* **33**, 1155-1162.

58. Ito, K., Urakawa, N. and Koike, H. (1982) Cardiovascular toxicity of palytoxin in anesthetized dogs. *Arch. Int. Pharmacodyn.* **258**, 146-154.

59. Halstead, B. W. (1988) Phylum Coelenterata (Cnidaria). *Poisonous and venomous marine animals of the world.* pp. 99-186, The Darwin Press Inc. Princeton, New Jersey.

60. Olivera, B. M., Hillyard, D. R., Rivier, J., Woodward, S., Gray, W. R., Corpuz, G., and Cruz, L. J. (1990) Conotoxins: Target peptide ligands from snail venoms. *Marine toxins: Origin, structure, and molecular pharmacology.* (Hall, S. and Strichartz, G. eds.) pp. 256-278, American Chemical Society, Washington DC.

61. Gray, W. R., Luque, A., Olivera, B. M., Barrett, J. and Cruz, L. J. (1981) Peptide toxins from *Conus geographus* venom. *J. Biol. Chem.* **256**, 4734-4740.

62. Lourdes, J., Cruz, W. R., Gray, D. Y. and Olivera, B. M. (1985) Conus venoms: A rich source of neuroactive peptides. *J. Toxicol.-Toxin* Rev. **4**, 107-132.

63. Kohn, A. J. (1966) The Ecology of *Conus* in Hawaii and California. *Ecol.* **4**, 1041-1046.

64. Rice, R. D. and Halstead, B. W. (1968) Report of fatal cone shell sting by *Conus geographus* L. *Toxicon* **5**, 223-228.

65. Yoshiba, S. and Sakurai, S.(1982) Comparative studies on the toxicities of venoms of five species of *Conus* and the effect of ligation as a treatment for *Conus* stings. *Jpn. J. Med. Sc. Biol.* **35**, 139-145.

66. Olivera, B. M., River, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. E., Woodward, S. R., Hillyard, D. R. and Cruz, L. J. (1990) Diversity of *Conus* neuropeptides. *Science* **249**, 257-263.

67. Myers, R. A., Cruz, L. J., Rivier, J. E. and Olivera, B. M. (1993) *Conus* peptides as chemical probes for receptors and ion channels. *Chem. Rev.* **93**, 1923-1936.

68. Endean, R., Parish, G. and Gyr, P. (1974) Pharmacology of the venom of *Conus geographus*. *Toxicon* **12**, 131-138.

69. Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A. and Gray, W. R. (1984) Purification and sequence of a presynaptic peptide toxin from *Conus geographus* venom. *Biochem.* **23**, 5087-5090.

70. Cruz, L. J., Gray, W. R., Olivera, B. M., Zeikus, R. D., Kerr, L., Yoshikani, D. and Moczydlowski, E. (1985) *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. *J. Biol. Chem.* **260**, 9280-9288.

71. Baslow, M. H. (1969) *Marine Pharmacology*. pp. 100-120, The Williams and Wilkins Co. Baltimore.

72. Kozloff, E. N. and Price, L. H. (1987) *Marine Invertebrates of the Pacific Northwest*. pp. 1-100, University of Washington Press, Seattle.

73. Friese, U. E. (1972) *Sea Anemones*. pp. 1-128, T. F. H. Publications Inc. Ltd. Hong Kong.

74. Der Maderosian, A. (1969) Marine pharmaceuticals. *J. Pharm. Sc.* **58**, 1-33.

75. Rathmayer, W. (1979) Sea anemone toxins: Tools in the study of excitable membranes. *Adv. Cytopharmacol.* **3**, 335-344.

76. Wood, J. and Lentz, T. L. (1964) Histochemical localization of amines in hydra and in the sea anemones. *Nature* **201**, 88-89.

77. Norton, R. S. (1991) Structure and structure-function relationships of sea anemone proteins that interact with the sodium channel. *Toxicon* **29**, 1051-1084.

78. Beress, L. (1982) Biologically active compounds from coelenterates. *Pure Appl. Chem.* **54**, 1981-1994.

79. Graff, D. and Grimmelikhuijzen, C. J. P. (1988) Isolation of <Glu-Gly-Leu-Arg-Trp-NH_2 (Antho-RWamide II), a novel neuropeptide from sea anemones. *FEBS Lett.* **239**, 137-140.

80. Blair, R. W., Peterson, D. J. and Bishop, V. S. (1978) The effects of anthopleurin-A on cardiac dynamics in conscious dogs. *J. Pharmacol. Exp. Ther.* **207**, 271-276.

81. Cariello, L., de Santis, A., Fiore, F., Piccoli, R., Spagnuolo, S., Zanetti, L. and Parente, A. (1989) Calitoxin, a neurotoxic peptide from the sea anemone *Calliactis parasitica*: amino acid sequence and electrophysiological properties. *Biochem.* **28**, 2484-2489.

82. Kem, W. R., Parten, B., Pennington, M. W., Price, D. A. and Dunn, B. M. (1989) Isolation, characterization and amino acid sequence of a polypeptide neurotoxin occurring in the sea anemone *Stichodactyla helianthus*. *Biochem.* **28**, 3483-3489.

83. Macek, P. (1992) Polypeptide cytolytic toxins from sea anemones (Actinaria). *FEMS Microbiol. Immunol.* **105**, 121-129.

84. Turk, T. (1991) Cytolytic toxins from sea anemones. *J. Toxicol.-Toxin Rev.* **10**, 223-262.

85. Maretic, Z. and Russel, F. E. (1982) Some epidemiological and clinical aspects of stings by anemone *Anemonia sulcata*. *Toxicon* **20**, 360.

86. Alsen, C., Peters, T. and Scheufler, E. (1982) Studies on the mechanism of the positive inotropic effect of ATX II (*Anemonia sulcata*) on isolated guinea pig atria. *J. Cardiovas. Pharmacol.* **4**, 63-69.

87. Norton, T. R., Shibata, S., Kashiwagi, M. and Bentley, J. (1976) Isolation and characterization of the cardiotonic polypeptide anthopleurin-A from the sea anemone *Anthopleura xanthogrammica*. *J. Pharm. Sci.* **65**, 1368-1374.

88. Beress, L., Beress, R. and Wunderer, G. (1975) Purification of three polypeptides with neuro- and cardiotoxic activity from the sea anemone *Anemonia sulcata*. *Toxicon* **13**, 359-367.

89. Mebs, D., Liebrich, M., Reul, A. and Samejima, Y. (1983) Hemolysins and proteinase inhibitors from sea anemones of the gulf of Aqaba. *Toxicon* **21**, 257-264.

90. Kem, W. R. and Dunn, B. M. (1988) Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon* **26**, 997-1008.

91. Norton, R. S., Bobek, G., Ivanov, B. J., Thomson, M., Fiala-Beer, E., Moritz, R. L. and Simpson, R. J. (1990) Purification and characterization of proteins with cardiac stimulatory and haemolytic activity from the sea anemone *Actinia tenebrosa*. *Toxicon* **28**, 29-41.

92. Shibata, S., Dunn, D. F., Kuchii, M., Kashiwagi, M. and Norton, T. R. (1974) Cardiac stimulant action of extracts of coelenterates on rat atria. *J. Pharm Sci.* **63**, 1332-1333.

93. Norton, T. R. (1981) Cardiotonic polypeptides from *Anthopleura xanthogrammica* (Brandt) and *A. elegantissima* (Brandt). *Fed. Proc.* **40**, 21-25.

94. Reimer, N. S., Yasunobu, C. L., Yasunobu, T. and Norton, T. R. (1985) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant anthopleurin-B. *J. Biol. Chem.* **260**, 8690-8693.

95. Zorec, R., Tester, M., Macek, P. and Mason, W. T. (1990) Cytotoxicity of equinatoxin II from the sea anemone *Actinia equina* involves ion channel formation and an increase in intracellular calcium activity. *J. Memb. Biol.* **118**, 243-249.

96. Scriabine, A., Van Arman, C. G., Morgan, G., Morris, A. A., Bennett, C. and Bohidar, N. R. (1979) Cardiotonic effects of anthopleurin A a polypeptide from a sea anemone. *J. Cardiovas. Pharmacol.* **1**, 571-583.

97. Shibata, S., Norton, T. R., Izumi, T. and Katsuki, S. (1976) A polypeptide (AP-A) from sea anemone (*Anthopleura xanthogrammica*) with potent positive inotropic action. *J. Pharmacol. Exp. Ther.* **199**, 298-309.

98. Shibata, S., Izumi, T., Seriguchi, D. G. and Norton, T. R (1978) Further studies on the positive inotropic effect of the polypeptide anthopleurin-A from a sea anemone. *J. Pharmacol. Exp. Ther.* **205**, 683-692.

99. Alsen, C.(1983) Biological significance of peptides from *Anemonia sulcata*. *Fed. Proc.* **42**, 101-108.

100. Vaughn, W. (1975) Classification of antidysrhythmic drugs. *Pharmac. Ther.* **B1**, 115-138.

101. Bergman, C., Dubois, J. M., Rojas, E. and Rathamayer, W. (1976) Decreased rate of sodium conductance inactivation in the node of Ranvier induced by a polypeptide toxin from a sea anemone. *Biochim. Biophys. Acta* **455**, 173-184.

102. Romey, G., Abita, J. P., Schweitz, H., Wunderer, G. and Lazdunski, M. (1976) Sea anemone toxin a tool to study molecular mechanisms of nerve conduction and excitation-coupling. *Proc. Natl. Acad. Sc. USA* **73**, 4055-4059.

103. Bernheimer, A. W. and Lai, C. Y. (1985) Properties of a cytolytic toxin from the sea anemone, *Stoichactis kenti*. *Toxicon* **23**, 791-799.

104. Bernheimer, A. W. and Avigad, L. S. (1982) Toxins of sea anemone *Epiactis prolifera*. *Arch. Biochem. Biophys.* **217**, 174-180.

105. Blumenthal, K. M. and Kem, W. R. (1983) Primary structure of *Stoichactis helianthus* cytolysin III. *J. Biol. Chem.* **258**, 5574-5581.

106. Simpson, R. J., Reid, G. E., Moritz, R. L., Morton, C. and Norton, R. S. (1990) Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and haemolytic protein from the sea anemone *Actinia tenebrosa*. *Eur. J. Biochem.* **190**, 319-328.

107., U., Macek, P. and Sedmak, B. (1990) The cytotoxic and cytolytic activity of equinatoxin II from the sea anemone *Actinia equina*. *Cell Biol. Int. Rep.* **14**, 1013-1024.

108. Giraldi, T., Ferlan, I. and Romeo, D. (1976) Antitumor activity of equinatoxin. *Chem.-Biol. Interactions.* **13**, 199-203.

109. Teng, C., Wang, J., Peng, H. and Ouyang, C. (1988) Edema-producing proteins isolated from *Trimeresurus mucrosquamatus* snake venom. *Toxicon* **27**, 899-905.

110. Khoo, K. S., Kam, W. K., Khoo, H. E., Gopalakrishnakone, P. and Chung, M. C. M. (1993) Purification and partial characterization of two cytolysins from a tropical sea anemone *Heteractis magnifica*. *Toxicon* **31**, 1567-1579.

111. Macek, P. and Lebez, D. (1988) Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L. *Toxicon* **26**, 441-451.

112. Shiomi, K., Takamiya, M., Yamanaka, H. and Kikuchi, T. (1986) Physicochemical properties of a lethal hemolysin isolated from the sea anemone *Anthopleura japonicum*. *Bull. Jpn. Soc. Sc. Fish.* **52**, 539-543.

113. Kem, W. R. (1988) Sea anemone toxins: structure and action. *Biology of Nematocysts.* (Hessinger, D. A. and Lenhoff, H. M. eds.) pp. 375-405, Academic Press Inc. New York.

114. Bernheimer, A. W. and Avigad, L. (1976) Properties of a toxin from the sea anemone *Stoichactis helianthus*, including specific binding to sphingomyelin. *Proc. Natl. Acad. Sci. USA* **73**, 467-471.

115. Doyle, J. W. and Kem, W. R. (1989) Binding of radiolabelled sea anemone cytolysin to erythrocyte membranes. *Biochim. Biophys. Acta* **987**, 181-186.

116. Varanda, W. and Finkelstein, A. (1980) Ion and non-electrolyte permeability properties of channels formed in planar lipid bilayer membranes by the cytolytic toxin from the sea anemone, *Stoichactis helianthus*. *J. Memb. Biol.* **55**, 203-211.

117. Belmonte, G., Pederzolli, C., Macek, P. and Menestrina, G. (1993) Pore formation by sea anemone cytolysin equinatoxin II in red blood cells and model lipid membranes. *J. Memb. Biol.* **131**, 11-22.

118. Hessinger, D. A. and Lenhoff, H. M. (1973) Assay and properties of the hemolysis activity of pure venom from the nematocysts of the acontia of the sea anemone *Aiptasia pallida*. *Arch. Biochem. Biophys.* **159**, 629-638.

119. Michaels, D. W. (1979) Membrane damage by a toxin from the sea anemone *Stoichactis helianthus*. 1. Formation of transmembrane channels in lipid bilayers. *Biochim. Biophys. Acta* **555**, 67-78.

120. Bernheimer, A. W., Avigad, L. S. and Kim, K. (1979) Comparison of metridiolysin from the sea anemone with thiol-activated cytolysins from bacteria. *Toxicon* **17**, 69-75.

121. Thomson, M., Moritz, R. L., Simpson, R. J. and Norton, R. S. (1987) Tenebrosin-A, a new cardiostimulant protein from the Australian sea anemone *Actinia tenebrosa*. *Biochem. Int.* **15**, 711-718.

122. Galettis, P. and Norton, R. S. (1990) Biochemical and pharmacological studies of the mechanism of action of tenebrosin-C, a cardiac stimulatory and haemolytic protein from the sea anemone, *Actinia tenebrosa*. *Toxicon* **28**, 695-706.

123. Budihna, M., Suput, D. and Macek, P. (1990) Cardiac arrhythmia and negative inotropic effect induced by equinatoxin II. *Int. Conf. IEEE Eng. Med. Biol. Soc.* **12**, 656-657.

124. Lafranconi, W. M., Ferlan, I., Russell, F. E. and Huxtable, R. J. (1984) The action of equinatoxin, a peptide from the venom of the sea anemone *Actinia equina* on the isolated lung. *Toxicon* **22**, 347-352.

125. Sket, D., Draslar, K., Ferlan, I. and Lebez, D. (1974) Equinatoxin, a lethal protein from *Actinia equina*. II. Pathophysiological action. *Toxicon* **12**, 63-68.

126. Mahnir, V. M., Shumlov, U. N., Kovalevskaya, A. M., Romanenko, L. A. and Grebelny, S. D. (1993) Evidence for several types of biologically active substances in north Pacific sea anemones. *Comp. Biochem. Physiol.* **106C**, 661-665.

127. Tabrah, F. L., Kashiwagi, M. and Norton, T. R. (1971) Antitumor activity in mice of four coelenterate extracts. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **5**, 420-423.

128. Norton, T. R. and Kashiwagi, M. (1972) Purification of a potent antitumor agent from a Tahitian sea anemone and methods of administration. Studies with Erlich ascite tumor in mice. *J. Pharm. Sc.* **61**, 1814-1817.

129. Quinn, R. J., Kashiwagi, M., Norton, T. R., Shibata, S., Kuchi, M. and Moore, R. E. (1974) Antitumor activity and cardiac stimulatory effects of constituents of *Anthopleura elegantissima*. *J. Pharm. Sc.* **63**, 1798-1800.

130. Batista, U., Macek, P. and Sedmak, B. (1986) The influence of equinatoxin II on V-79-379 a cell line. *Period. Biolog.* **88**, 97-98.

131. Batista, U., Jezernik, K., Macek, P. and Sedmak, B. (1987) Morphological evidence of cytotoxic and cytolytic activity of equinatoxin II. *Period. Biolog.* **89**, 347-348.

132. Sedmak, B., Skrk, J. and Lebez, P. (1986) Cytolysin inhibits growth of melanoma and sarcoma cell *in vivo*. *Period. Biolog.* **88**, 198.

133. Mathias, A. P., Ross, D. M. and Schachter, M. (1960) The distribution of 5-hydroxytryptamine, tetramethylammonium, homorine and other substances in sea anemones. *J. Physiol.* **151**, 296-311.

134. Kettridge, J. S., Isbell, A. F. and Hughes, R. R. (1967) Isolation and characterization of the N-methyl derivatives of 2-aminoethylphophonic acid from the sea anemone *Anthopleura xanthogrammica*. *Biochem.* **6**, 289-294.

135. Howe, N. R. and Sheikh, Y. M. (1975) Anthopleurine: a sea anemone alarm pheromone. *Science* **189**, 386-388.

136. Zelnik, R., Haaraguchi, M., Matida, A. K., Lavie, D., Frolow, F. and Weis, A. L. (1986) X-Ray molecular structure of caissarone, a novel purine derivative from the sea anemone *Bunodosoma caissarum* Correa 1964. *J. Chem. Soc. Perkin Trans.* **1**, 2051-2053.

137. Chakrabarty, M., Batabyal, A. and Barua, A. K. (1994) New ceramides from the hypotensive extract of a sea anemone *Paracondylactis indicus*. *J. Nat. Prod.* **57**, 393-395.

138. Mahnir, V. M., Kozlovskaya, E. P. and Kalinovsky, A. I. (1992) Sea anemone *Radianthus macrodactylus* - a new source of Palytoxin. *Toxicon* **30**, 1449-1456.

139. Ishikawa, Y., Onodera, K. and Takeuchi, A. (1979) Purification and effect of the neurotoxin from the sea anemone *Parasicyonis actinostoloides*. *J. Neurochem.* **33**, 69-73.

140. Shiomi, K., Tanaka, E., Yamanaka, H. and Kikuchi, T. (1985) Isolation and characterization of a lethal hemolysin in the sea anemone *Parasicyonis actinostoloides*. *Toxicon* **23**, 865-874.

141. Kem, W. R., Dunn, B. M., Parten, B. Pennington, M. and Price, D. (1985) The unconventional amino acid sequence of the major neurotoxin polypeptide of the sun sea anemone *Stoichactis helianthus*. *Fed. Proc.* **45**, 1795.

142. Hessinger, D. A., Lenhoff, H. M. and Kahan, L. B. (1973) Hemolytic, phospholipase A and nerve-affecting activities of sea anemone nematocyst venom. *Nature New Biol.* **241**, 125-127.

143. Bernheimer, A. W. and Avigad, L. (1978) A cholesterol-inhibitable cytolytic protein from the sea anemone *Metridium senile*. *Biochim. Biophys. Acta* **541**, 96-106.

II. Proposed Research

2.1 Preamble

Heart failure is an abnormality in cardiac function that results in the inability of the heart to pump blood commensurate with the body's need at rest and during normal cycle. Failure then develops from myocardial contraction such as occurs in hypertension, ischemic heart disease and congenital heart disease.

Congestive heart failure (CHF) is a common disorder with an extremely high mortality rate. It affects over 4 million people in North America, with approximately 250,000 new cases each year.[1] CHF involves a cycle whereby the heart begins to fail and the body initiates a series of reflexes making it harder for the heart to work. Since the heart is already diseased, it fails even further until mortality occurs.

Until a decade ago, there was no effective treatment for CHF that reduced mortality associated with the disease. Despite the overall reduction of cardiovascular mortality, the incidence of death as a result of CHF is steadily increasing. For many years, the cardiac glycosides have been the only available cardiotonic agent for heart failure therapy, but their use is limited by their toxicity and narrow margin of safety. Other limitations includes their vasoconstrictor effect and their efficacy. Today, the new cardiovascular drugs that are available control the symptoms of the disease but are not

curative. Medicinal chemists have designed and synthesized new compounds with greater potency, efficacy and fewer side effects than ever before. Despite all of this, there is still no available cure for heart failure. This absence of safe positive inotropic agents has initiated considerable efforts devoted to the search for novel cardiotonic agents. One of the areas being harnessed is the sea.

While examining extracts of different species of cnidarians for antitumor activity, Shibata and his co-workers[2] discovered that extracts of nine out of the twelve species of sea anemones tested showed varying degrees of cardiotonic activity. They subsequently purified AP-A, AP-B and AP-C from *Anthopleura xanthogrammica* and *A. elegantissima* as the active components. Since then other cardiac stimulatory polypeptides have been isolated from unrelated sea anemone species.[4] Today AP-A has been shown in several studies to increase the release of calcium from the SR^3 as a result of an accumulation of intracellular sodium due to delayed gating of the sodium channel.[5]

Of at least 22 species of sea anemones identified and known to exist in the colder waters of the west coast of Canada, only two species (*A. xanthogrammica* and *Metridium senile*) have been sampled for cardiotonic activitiy.[87,120] The subject matter of this thesis was therefore to screen sea anemone species prevalent along the west coast of Canada and identify species with potent cardiotonic activity with the intent of isolating and characterizing the active constituents.

2.2 Objectives.

Specific objectives of this thesis were:

(1) To screen as many species of sea anemones as could be collected from the west coast of Canada for cardiac stimulatory activity using rat and guinea pig atrial and ventricular tissues. To determine which animal tissue was the most sensitive to the extracts.

(2) To determine among the sea anemones collected, which possessed the most potent cardiostimulant activity that had not been investigated previously.

(3) To develop a method of separation and purification of the active substance(s) using different chromatographic techniques.

(4) To characterize biochemically as far as possible the active substance(s).

(5) To screen the crude extracts and pure active substance(s) for other biological activities.

(6) To evaluate the effects of the active substances, using different *in vitro* and *in vivo* pharmacological methods, in an attempt to determine their mechanisms of action.

Bibliography

1. Ruffolo, R. R. (1995) Cardiovascular drugs past, present and future. *Pharm. News* **2**, 13-15.

2. Shibata, S., Dunn, D. F., Kuchii, M., Kashiwagi, M. and Norton, T. R. (1974) Cardiac stimulant action of extracts of coelenterates on rat atria. *J. Pharm Sc.* **63**, 1332-1333.

3. Shibata, S., Izumi, T., Seriguchi, D. G. and Norton, T. R. (1978) Further studies on the positive inotropic effect of the polypeptide anthopleurin-A from a sea anemone. *J. Pharmacol. Exp. Ther.* **205**, 683-692.

4. Thomson, M., Moritz, R. L., Simpson, R. J. and Norton, R. S. (1987) Tenebrosin-A, a new cardiostimulant protein from the Australian sea anemone *Actinia tenebrosa*. *Biochem. Int.* **15**, 711-718.

5. Hashimoto, K., Ochi, R., Hashimoto, K., Inui, J. and Muira, Y. (1980) The ionic mechanism of prolongation of action potential duration of cardiac ventricular muscle by anthopleurin-A and its relationship to the inotropic effect. *J. Pharmacol. Exp. Ther.* **215**, 479-485.

III. MATERIALS AND METHODS

3.1 Sea anemones

The choice of which sea anemones to use for this research work was based on the following criteria:

1. Species that were common to the west coast of Canada which had not been investigated for cardiac stimulatory activity.

2. The availability and abundance of these species within the region where they were being collected, in this case Vancouver, BC.

3. Proper identification of the sea anemones.

4. Their size and whether collection and biological activity would be affected by seasonal variation. Preliminary experiments to determine this had previously been evaluated by summer students of the late Dr. M. W. Wolowyk prior to the start of this research.

Eleven species of sea anemones (Table 3.1) that met the above criteria were identified by the marine biologist of Seacology and confirmed from Kozloff.[1] Sea anemones (3.5 g - 1.5 kg) were collected from Barkley Sound, Vancouver by *Seacology, a collecting agency. Two separate collections were made at different times of the year (January and June). After collection, individual species were bagged in sea water and shipped in ice by air the next day to Edmonton. On arrival in Edmonton, they were weighed and bagged in ziplock bags, labeled and kept at -70 °C until required for use.

---

* 3025 Sunnyhurst Rd. North Vancouver BC V7K 2G4

Table 3.1 Sea anemone species used in the research

Sea anemone	Family	Activity	Reference
Anthopleura elegantissima (Brandt)	Actiniidae	cardiotonic (AP-C)	(2)
A. xanthogrammica (Brandt)	"	cardiotonic (AP-A, AP-B)	(3, 4)
Corynactis californica (Calgren)	Sagartiidae	-	(5)
Epiactis prolifera (Verrill)	Actiniidae	Hemolytic (Epiactin A,B,C)	(6, 7)
Metridium senile L.	Metridiiddae	Hemolytic (Metridiolysin)	(8)
Pachycerianthus fimbratus	Unknown	-	-
Stomphia didemon (Siebert)	Corallimorphidae	-	-
Urticina coriacae (Cuvier)	Actiniidae	-	-
U. crassicornis (O. F. Muller)	"	-	-
U. lofotensis (Danielson)	"	-	-
U. piscivora (Sebens and Laakso)	"	-	-

(-) indicates that activity of the sea anemone has never been investigated.

Activity: in parentheses are the names of partially or completely characterized polypeptide toxins and their known activities. Taxonomic authority for *P. fimbratus* is unknown at this time.

3.2 Extraction of sea anemones

Before extraction, sea anemones were thawed and cut into small pieces < 2 cm^2. They were homogenized with a polytron in 30 % (v/v) ethanol for 10 min. at 4 °C. The mixture was centrifuged at 3,000 *g* using a refrigerated floor centrifuge (Sorvall RC 58) for 20 min. and the pellet washed with ethanol. Supernatants were pooled and lyophilized to give a water-soluble dry powder, which was stored at -70 °C.

3.3 *In vitro* experiments

3.3.1 Physiological solutions

For *in vitro* assays involving heart and smooth muscle preparations, Krebs-Hensleit buffer of the following composition (mM) was used; NaCl, 118; D-glucose, 11; KH_2PO_4, 1.18; KCl, 1.74; $MgSO_4.7H_2O$, 2.41; $CaCl_2.2H_2O$, 2.52 and $NaHCO_3$, 25; pH 7.4.

For *in vitro* hemolytic experiments, phosphate buffered saline (PBS) of the composition (mM) was used: NaCl, 136; KCl, 2.7; Na_2HPO_4, 8.0 and KH_2PO_4, 1.5, pH 7.4.

For scanning electron microscope studies, Millonigs phosphate buffer of the composition (mM) was used: $Na_2HPO_4.H_20$, 0.25; $Na_2HPO_4.7H_2O$, 6.2 and NaCl, 0.3; pH 7.2.

3.3.2 Cardiac stimulatory activity

To determine which animal tissue was the most sensitive for this assay, rat and guinea-pig left atrial tissue and guinea pig ventricular tissues were assessed. Once the tissue was identified (Section 4.2.3), it was then used throughout the isolation and purification process.

Male Hartley strain guinea-pigs (350-500 g, Charles River Canada Inc., St. Constant, Quebec) and Sprague Dawley rats (200 - 300 g, University of Alberta Animal Services) were used. The animals were anesthetized in a closed chamber with CO_2 generated from dry ice and water. The thoracic cavity of the animals was opened to expose the thoracic organs. Working as quickly as possible, the heart was excised and immediately immersed in Krebs-Hensleit buffer at 37 °C. Extraneous tissues attached to the atria were dissected and the left atrium separated from the right. The ventricles were cut into strips about 2 mm thick.

A thread was attached to each end of the left atrium and ventricle respectively. The bottom thread was used to anchor the tissue to a platinum stimulating electrode (25 gauge platinum wires, embedded 1 mm apart in plastic). The tissues were suspended in a 10 ml organ bath (Figure 3.1) containing the buffer. They were then connected by the upper thread to a force displacement transducer (Grass model FT03). The temperature of the bath was maintained at 37 °C, the tissues continuously aerated with 95 % O_2 - 5 % CO_2 and electrically driven at a constant rate of 1.5 Hz. All tissues were equilibrated for

60 min. and movements recorded isometrically on an eight channel polygraph (Grass model 7D). Changes in contractile force were expressed as percentage increase over the control period immediately preceding addition of test compounds and a known positive inotrope, Isoproterenol (INA). A non cumulative method of addition was used.

3.3.3 Guinea-pig ileal longitudinal smooth muscle (GPILSM)

The same strain of guinea-pig as above was used. The method used to isolate the desired organ was that of Rang.[9] The anesthetized animal was laid on its back and a longitudinal mid-line incision made in the abdominal wall. The caecum was lifted, shifted to the right side of the animal to expose the ileo-caecal junction. The ileum was cut a few centimeters above this junction and freed of mesenteric attachments for about 20 cm. A second cut was made to isolate the section. The section was stretched over a glass rod of approximately equal diameter (1 mm) and immersed in Krebs-Hensleit buffer maintained at 37 °C. Two incisions were made on either side of the line of mesenteric attachment with a blunt scalpel blade. A cotton tipped applicator (Q-tip) was used to separate the longitudinal smooth muscle by brushing it away from the underlying tissue. Sections (1.5 - 2 cm) were prepared by tying each end with a piece of thread and using the thread to suspend the tissue in a 10 ml organ bath (Figure 3.1). One thread was fixed to the bottom of the bath to anchor the tissue and the other to a force displacement transducer connected to a polygraph as already described. An initial tension of 500 mg was applied to each tissue which was then equilibrated for 45 min. with buffer change every 15 min. The

resting tension declined during the equilibration period and was subsequently adjusted to 250 mg at the start of the experiment. The chart speed was set at 2.5 - 5.0 cm/min. to observe changes in tension during the experiment.

The contractile effect of test compounds were examined using ACh as a control. After an equilibration period, contractile effects were measured by exposing the tissues to test doses of the agonists.

To test the effect of blocking agents, atropine (10^{-8} M) and TTX (0.05 μg/ml) on the contractile action of test substances, the tissues were first equilibrated with the antagonist for 5 min., after which a dose of the agonist was added.

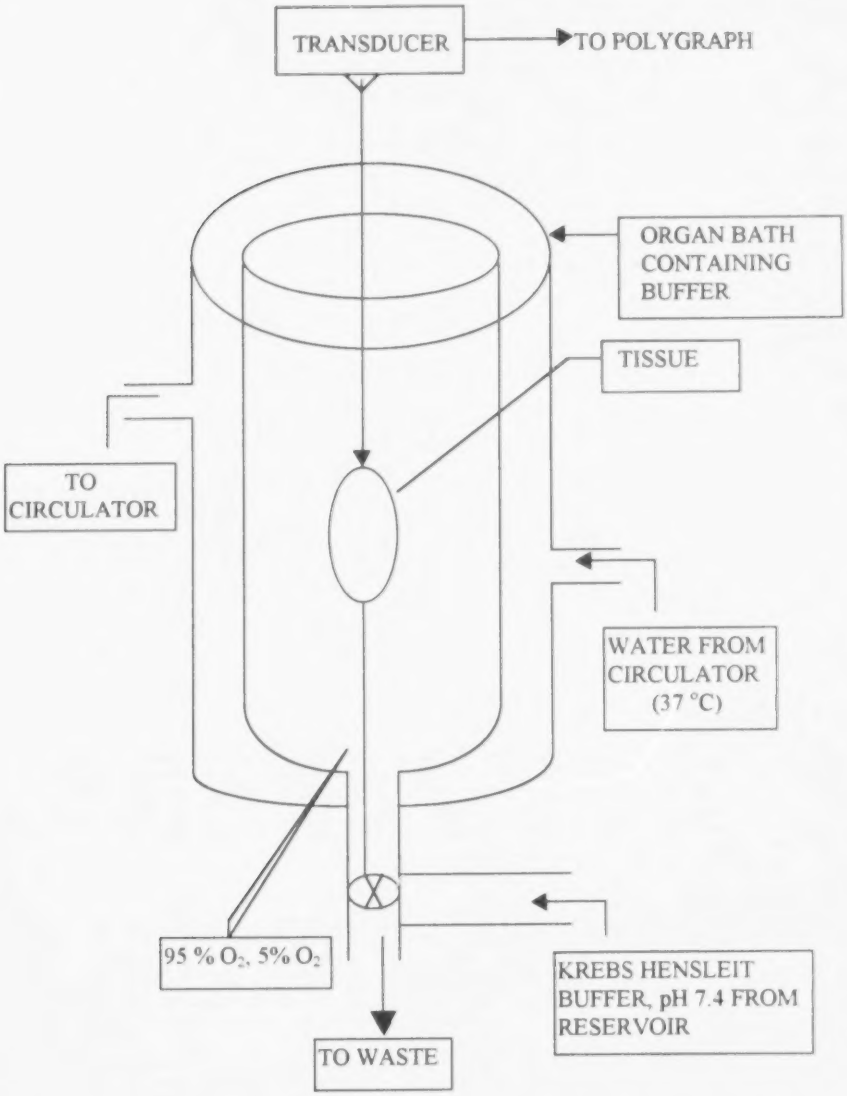


Figure 3.1 Isolated tissue set-up for smooth muscle and right atrium. Left atrium was attached to a stimulating electrode and suspended in a similar manner. This set-up was used for cardiac stimulatory and smooth muscle bioassays

3.3.4 Hemolytic activity

The lysis of washed erythrocytes of different mammalian species (rat, guinea pig, pig, dog and human) was measured by the release of hemoglobin, using the method of Hessinger and Lenhoff.[10] Blood (5 - 10 ml) was mixed with 0.5 ml heparin (100 units/ml) (Sigma Chemical Co. St. Louis, MO), in chilled PBS. The mixture was centrifuged at 3000 rpm with a bench centrifuge (Damon, IEF, Damon, USA) for 5 min. at room temperature. The plasma and buffy coat were removed and the cells washed three times with 15 ml PBS. The packed cell volume was determined with a hematocrit and the cells resuspended to a 0.9 % hematocrit in chilled PBS. The samples to be assayed were resuspended in 100 µl PBS and to it was added 2 ml 0.9 % erythrocytes, mixed and incubated for 30 min. at 37 °C with gentle shaking. The tubes were centrifuged at room temperature for 5 min. and the absorbance of the supernatant measured at 540 nm to detect released hemoglobin. Total hemolysis was achieved by resuspending 1 ml erythrocyte in 3 ml distilled water. This corresponded to an A_{540} of about 1.2. Controls consisted of 100 µl PBS in place of the test compounds. All assays were carried out in triplicate.

The effect of the phospholipid sphingomyelin (2.5 and 25 µg/ml) on the hemolytic action of one test compound (UpI) was evaluated. UpI (10^{-5} M) was incubated with sphingomyelin at 37 °C for 1 h, before the mixture was assayed for hemolytic activity as already described.

3.3.5 Cytotoxic activity

The *in vitro* cytotoxic action of crude extracts and proteins from sea anemones was evaluated by Synphar Laboratories Inc. in Edmonton. It was carried out using a modification of the crystal violet assay.[11] Three cell lines, KB cells (ATCC CCL 17, oral human epidermoid carcinoma), L1210 (mouse lymphocyte leukemia), and HEL 299 cells (human embryonic lung diploid) were used.

The cells were cultivated in Eagles minimum essential medium supplemented with 10 % calf serum and incubated at 37 °C in a humidified CO_2 atmosphere, for preparation of cell stock. The cells were counted using a Neubauer hemocytometer and seeded in 96-well plates at 100 µl of $3x10^4$ cells/ml and cultured for 24 h. Test compounds were diluted and 100 µl added to give a final concentration of 1 - 200 µg/ml. Each test was done in triplicate. Control wells were identical but with no test substance present. The cells were cultured for three days, fixed with 20 µl 25 % (w/v) glutaraldehyde for 15 min., washed with water and dried. They were then stained with 100 µl 0.05 % crystal violet for 15 min., washed again and dried. The wells were eluted with 100 µl 0.05 M NaH_2PO_4/ethanol (1:1 by vol.) and absorbance read at 540 nm on a multiscan spectrophotometer. The concentration at which 50 % toxicity (TC_{50}) was achieved was calculated using the formula below:

$$\frac{\text{\% inhibition} > 50\ \% - 50\ \%\ \text{inhibition}}{\text{\% inhibition} > 50\ \% - \text{\% inhibition} < 50\ \%}$$

3.3.6 Antifungal activity

Screening for antifungal activity was also carried out by Synphar using the diffusion technique in petri dishes.[12] Test cultures were *Candida albicans* ATCC 14053 and *Aspergillus niger* PLM 1140. *C. albicans* was grown on Sabouraud medium while *A. niger* was grown on RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS. Sea anemone extracts (1 - 200 μg/ml) were introduced into holes (≈ 8 mm diameter) in the agar medium. The petri dishes were placed in an incubator and allowed to stand for 4 h at 30 °C to improve diffusion of substrates into the agar. The agar plates were then seeded with test microorganisms (suspension in saline) and incubated for 24 - 36 h at 37 °C. Following incubations, the diameter of the zones of inhibition was measured.

3.4 Separation and purification of cardiotonic proteins

All solvents for chromatography were HPLC grades purchased from Fischer Scientific, Edmonton, AB. Other reagents were purchased from either Aldrich Chemical Co. Inc., Milwaukee Wis. and/or Sigma Chem. Co. Powdered gels were purchased from Pharmacia Biotechnology, Montreal Quebec.

3.4.1 Gel filtration chromatography

3.4.1.1 Preparation of gel

The medium used for this process was cross-linked dextran gel, Sephadex G-50 M. It was prepared by mixing 100 g of the dry powder with 500 ml distilled water to give a slurry. The slurry was heated to 100 °C. This allowed the gel to swell within a few hours and removed any dissolved air. The gel was allowed to stand at 4 °C until the slurry had settled and the liquid decanted off to remove fine particles. The gel was then equilibrated in the desired buffer at the same temperature overnight. The slurry was de-aerated with a sonicator for 20 min. and carefully poured into a vertical column. The column was equilibrated with buffer overnight at a flow rate of 1 ml/min., after which the column was ready for use.

3.4.1.2 Standardization of gel filtration column

The column used was 120 x 3 cm (BioRad, CA USA). Before standardization, a colored test sample (Blue dextran) was allowed to flow thorough the column to ensure the absence of air bubbles and or a tailing effect. Gel chromatography standards, blue dextran (2,000 kDa), trypsin inhibitor (20 kDa) and bromophenol blue (692 Da) were used to standardize the column. Each was run through the column, 1 ml fractions collected and monitored at 540 nm. This was used to determine the void volume (V_o) of the column, the

elution volumes (V_e) of the fractions eluted and the total volume (V_t) of the column. The column was washed with buffer and equilibrated for use.

3.4.1.3 Separation of crude extracts

The crude extracts (1-1.5 g per batch) were separated in 65 batches on the above column, which contained the gel (V_o 185 ml; V_t 1000 ml), equilibrated with 0.1 M NH_4HCO_3 containing 100 nM NaN_3 to prevent microbial growth. The crude extract was dissolved in 4 ml of buffer, mixed gently and centrifuged for 5 min. at 3000 rpm. The supernatant was placed onto the gel and eluted with the same buffer at 4 °C. The flow rate of the column was 1 ml/min. and 5 ml fractions were collected and monitored at 280 nm. Pooled fractions were assayed for cardiac stimulatory activity using rat left atria. Active fractions were lyophilized, weighed and stored at -70 °C.

3.4.2 Ion exchange chromatography

3.4.2.1 Choice of ion exchanger

Several kinds of ion exchangers were used to determine which was most suitable for separation of active cardiac stimulatory fraction obtained from gel filtration chromatography.

Anion exchange chromatography was carried out using Fast Protein Liquid Chromatography FPLC (Pharmacia). The sample (200 μg) was dissolved in 1 ml 50 mM

Tris-HCl pH 8.0 (buffer A), centrifuged and the supernatant injected onto the anion exchange column Mono Q HR 5/5. The sample was separated using buffer A and a linear gradient (0-100 %) of 0.5 M NaCl (buffer B), over a 30 min. period. The flow rate of the column was 1 ml/min. and fractions detected at 280 nm. Fractions (1 ml) were collected, pooled and assayed for cardiac stimulatory activity.

The different cation exchangers used were Mono S HR 5/5 for the FPLC system, CM-Sephadex C-25, and SP-Sephadex C-25. For FPLC, the buffer system was 40 mM phosphate buffer with pH ranging from of 4 - 6 respectively (buffer A) and a linear gradient, 0 - 100 %, 0.5 M NaCl (buffer B) over a 30 min. period. Flow rate, detection and fraction size collected were the same as those used for the anion exchange column.

For the Sephadex cation exchangers, 5 g of the powdered gel was mixed with sodium acetate buffer at pH ranging from 2 - 7.5, mixed intermittently and allowed to stand at room temperature until the slurry attained the required pH desired. The gel was then poured into 5 ml syringes (used as mini columns) and equilibrated with the appropriate buffer. The sample to be separated (50 µg) was dissolved in 250 µl of the buffer and applied to the column and separated using increasing concentrations of the sodium acetate (0.01 - 1 M) at the desired pH.

3.4.2.2 Separation of active fractions by cation exchange chromatography

The cation exchanger and conditions found to be most suitable for separation was SP - Sephadex C-25, sodium acetate buffer, pH 4.5. The active fraction (1.8 g) was

dissolved in 15 ml 0.01 M sodium acetate pH 4.5, mixed thoroughly and centrifuged in Eppendorf microcentrifuge tubes for 5 min. The supernatant was applied to a column (23 x 3 cm, Pharmacia). The flow rate of the column was 1 ml/min. and fractions were eluted using increasing concentrations of sodium acetate (0.01 - 1 M) pH 4.5. Fractions (2 ml) were collected and absorbance monitored at 280 nm. Pooled fractions were assayed for cardiac stimulatory activity, lyophilized, weighed and stored at - 70 °C.

3.4.3 Reverse phase chromatography (RpC)

Preparative RpC was performed on a SynChropak RP, C_8 column, 200 x 1 cm (Synchrom Inc. IN USA). The active fraction from cation exchange (25 mg) was dissolved in 5 ml 0.05 % (v/v) aqueous TFA (buffer A), centrifuged in Eppendorf tubes for 5 min. and the supernatant filtered through a Millex HA 0.45 µm filter (Millipore, MA USA) before injecting onto the column. The fractions were eluted using a linear gradient of 0.05 % TFA in acetonitirile, with a flow rate of 2 ml/min. Fractions (2 ml) were collected, monitored at 210 nm, pooled, assayed for cardiac stimulatory activity, lyophilized and stored at -70 °C.

For the separation of protein variants (isotoxins), the active fraction (4.2 mg) was dissolved in 5 ml 0.05 % TFA, centrifuged and filtered as described above. The supernatant was injected onto an Aquapore RP-300 C_8 column, 220 x 4.6 mm. All other conditions for separation were the same as above.

Analytical RpC was carried out to confirm the purity of the active protein(s) and to further remove residual minor impurities. It was performed on a Zorbax 300 SB C_8 column, 15 x 2.1 mm (Zorbax, USA) using the same solvent conditions as the preparative RpC. The flow rate of the column was 250 µl/min. and peaks were monitored at 210 nm.

3.4.4 Physicochemical characterization of proteins

All reagents used for this characterization were electrophoresis grade purchased either from BioRad, Pharmacia and or Boerhringer Mannheim Biomedicals, Laval, Quebec. All sequencing reagents were purchased from Applied Biosystems, Forster City, CA, USA.

3.4.4.1 SDS-PAGE

To monitor fractions obtained during the separation and purification procedure, the automated PhastGel electrophoresis system (Pharmacia LKB, Sweden) was used. For estimation of the molecular mass of proteins and for confirmation of the specific protein band possessing cardiac stimulatory activity, manual SDS-PAGE was used.

For automated PhastGel electrophoresis the following conditions were used. The protein sample (10 µg/ml) contained the following: 1.5 µl buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 1 µl 20 % (w/v) SDS, 1 µl 50 % β-mercaptoethanol and 1 µl 0.1 %

(w/v) bromophenol blue. The mixture was heated for 5 min. at 100 °C, cooled at room temperature and 1 µl applied to the PhastGel 1/8 sample applicator. The resolving gel was an 8 - 25 % gradient mini gel (43 x 50 x 0.45 mm) and SDS PhastGel buffer strips. The gels were run at 250 V, 15 °C for 30 min. They were then stained with 0.2 % (v/v) PhastGel Blue R in aqueous acetic acid and destained with a mixture of methanol/acetic acid/water (3:1:6 by volume). Gels were preserved in a solution of 10 % (v/v) glycerol.

For manual SDS-PAGE, a 15 % polyacrylamide gel (85 x 100 x 0.5 mm) was used at all times. It was carried out using the method of Laemmli.[13] Protein samples (25 µg/ml) were resolved in the presence and absence of β-mercaptoethanol under constant voltage (200 V) for 4 - 6 h. The gels were stained for 4 h with 0.025 % (w/v) commassie blue in 50 % (v/v) ethanol and 10 % (v/v) acetic, and destained overnight in a mixture of 10 % methanol and 7 % acetic acid in distilled water.

3.4.4.1.1 Determination of molecular mass of proteins

Protein samples were resolved by manual SDS-PAGE carried out in both reducing and non-reducing conditions as already described. A mixture of the polypeptides and proteins of known molecular mass ran on the same gel was used for estimating the molecular mass of the isolated proteins. The molecular markers (Pharmacia) were, β-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa) and phosphorylase b (94 kDa). After development

of the gels, the movement of the markers (mm) from the cathode end was measured together with that of the purified proteins. Calibration curves were generated using the distance moved versus the molecular weights. Apparent molecular weights of the purified proteins were interpolated from the calibration curves.

3.4.4.1.2 Confirmation of cardiac stimulatory activity by SDS-PAGE

Protein samples (100 µl, 10^{-7} M) were resolved on a 15 % gel in non-reducing conditions as already described. On the left end of the gel were placed molecular weight markers, with protein samples in all other wells. After completion of electrophoresis, a thin strip of gel with the protein was cut together with the strip containing the molecular markers. They were stained for 2 h and destained as already described. The protein strip was used to locate the position of the protein on the rest of the gel while the molecular marker strip was used to locate the positions of the different standard proteins. The gel was then cut into horizontal strips based on the positions of the molecular markers and the proteins. Each strip was homogenized in 2 ml Krebs-Hensleit buffer, pH 7.4, centrifuged in Eppendorf tubes and the supernatant tested on rat left atria for cardiac stimulatory activity as described in Section 3.3.2.

3.4.4.2 Estimation of isoelectric points (pI) of proteins

Isoelectric focusing was carried out using the automated PhastGel system. The gel used was the PhastGel IEF 3 - 9. Protein samples (10 μg) were dissolved in 100 μl distilled water and 1μl applied to the PhastGel applicator, with methyl red as the tracking dye. The gels were run at 200 V, 15 °C for 30 min. They were developed by first fixing with 20 % (w/v) trichloroacetic (TCA) for 5 min., washed and destained with a mixture of methnol/acetic acid/water (3:1:6 by volume). The gels were stained with 0.2 % PhastGel Blue R in the destaining solution containing 0.1 % $CuSO_4$. The pI markers used were trypsin inhibitor (pI 4.6), β-lactoglobulin (pI 5.1), carbonic anhydrase I (pI 6.6), myoglobin (pI 7.2), L-lactic dehydrogenase (pI 8.6) and trypsinogen (pI 9.3) (Sigma Chem Co.). The distance moved by each polypeptide was measured from the cathode (mm) and a calibration curve of pI versus distance generated. The pI's of the proteins were then estimated from this.

3.4.4.3 Estimation of protein content

The protein content of crude extracts and isolated protein solutions was determined using the BioRad protein assay kit, as described by the manufacturer. Crude extracts (1 mg) and isolated proteins (100 μg) were dissolved in 1 ml and 200 μl distilled water respectively, centrifuged in Eppendorf tubes and 100 μl of the supernatant added to

glass tubes. To each tube was added 5 ml of the dye solution (commassie blue) and they were allowed to stand for 30 min. at room temperature. The absorbancies were measured at 595 nm with BSA as standard. Each test was done in triplicate. Standard curves for BSA (1-150 mg/ml and 10-450 μg/ml respectively) were generated and the protein contents of the test compounds interpolated.

3.4.4.4 Amino acid analysis

This was carried out with the assistance of Mr. Jack Moore of the Alberta Peptide Institute. Analyses were performed on a Beckman model 6300 analyzer equipped with a Hewlett Packard (HP) 3390 integrator. Samples (100 μg) were hydrolyzed at 110 °C for 24 h *in vacuo* with gaseous HCl generated from 6 M HCl containing 0.1 % w/v phenol. For cysteine analysis, the method of Spencer and Wold[14] was employed whereby the above hydrolysis was carried out in the presence of DMSO.

3.4.4.5 N-terminal sequence analysis

This was carried out by Mr. Jack Moore of the Alberta Peptide Institute. Automated amino acid analysis was performed on an HP model HPG 1000A sequencer. Pure intact proteins (50 μg) were dissolved in 50 % acetonitrile and subjected to automatic Edman degradation runs with the sequencer. Protein samples were loaded onto an HP biphasic sequencing column. The amino acid phenylthiohydantoins (PTH-amino

acids) were analyzed on an HP model 1090 series II HPLC fitted with a PTH-AA column (250 x 2.1 mm). The runs were terminated at a step after which the PTH-amino acid yields were insufficient to be identified.

3.4.5 Scanning electron microscopy (SEM) studies

The effects produced by hemolytic proteins on the membranes of erythrocytes were evaluated using SEM. Rat and guinea pig erythrocytes were used for this purpose. Erythrocytes were collected and treated as already described (section 3.3.4) and a 7 % suspension prepared. The test protein (50 µl of 10^{-5} M) was added to 0.2 ml of the erythrocyte suspension and the mixture incubated for 30 min. at 37 °C during which time lysis occurred. The mixture was centrifuged at 3000 rpm for 20 min., washed three times with PBS and the supernatant discarded. The sediments were prepared using the standard protocol for SEM described below.

The sediments were fixed with 2.5 % glutaraldehyde in Millonigs buffer pH 7.2 (Section 3.3.1) at room temperature for 1 h. They were washed in the same three times for 10 min. respectively after which they were fixed in 1 % (w/v) OsO_4. The sediments were then washed in distilled water and dehydrated with grades of ethanol ranging from 50 % (v/v) to absolute. They were then dried at 31 °C for 5 - 10 min., dried overnight in a dessicator and sputter coated with gold (Edwards model S150 B sputter coater). The specimens were observed using the scanning electron microscope (Hitachi model S-2500).

Control membranes consisted of erythrocytes lysed with distilled water and treated as described.

3.4.6 Pathophysiological studies

3.4.6.1 *In vivo* measurement of blood pressure (BP) and heart rate (HR)

The effect of the extracts and proteins isolated from *U. piscivora* on BP and HR was studied in anaesthetized rats. Rats (Sprague Dawley, 400 - 500 g, University Animal Services), were anesthetized with urethane from Sigma Chem. Co. (25 %, w/v, 0.5 ml/100 g). The trachea was first cannulated, and the carotid artery exposed in the neck region and cannulated via a polyethylene cannula (PE 50) which was then connected to a previously cannulated pressure transducer (Statham P 23 Dd) for BP measurements. The jugular vein was exposed, cannulated and injections of test compounds made through this route. Chart speed was set at 25 mm/min. for BP and 25 mm/sec for HR measurements respectively. Three rats were used for each test. Doses of test compounds, extracts (0.5 - 100 μg/kg) and proteins (0.0125 - 20 μg/kg) were injected in ascending order and the response allowed to return to baseline before injection of the next dose.

The effect of atropine (3 mg/kg) on crude extracts and proteins was also investigated using the method of Huang and Mir.[15] To study the effect of atropine, the

animals were first injected with atropine, then anesthetized before administration of test compounds. Each animal was autopsied.

3.4.6.2 Histological examinations

This was carried out with the assistance of Dr. Nick Nation of the Animal Lab Services. Tissues to be examined were removed from each animal and placed in 10 % neutral buffered formalin and fixed for 24 h. Following fixation, the tissues were trimmed into histological cassettes and infiltrated with paraffin in an automatic tissue processor. Paraffin-infiltrated tissues were processed into blocks and each block sectioned at 5 μ. Sections were stained with hematoxylin and eosin and examined microscopically. The brain, heart, lung, liver, kidney and skin were examined, to determine any structural damage to the tissues.

Bibliography

1. Kozloff, E. N. and Price, L. H. (1987) *Marine Invertebrates of the Pacific Northwest.* pp. 1-100. University of Washington Press, Seattle.

2. Norton, T. R., Kashiwagi, M., and Shibata, S. (1978) Anthopleurin A, B and C, cardiotonic polypeptides from the sea anemones *Anthopleura xanthogrammica* (Brandt) and *A. elegantissima* (Brandt). *Drugs and Food from the Sea.* (Kaul, P. N. and Sinderman, C. J. eds.) pp. 37-50, University of Oklahoma Press, Oklahoma.

3. Norton, T. R., Shibata, S., Kashiwagi, M. and Bentley, J. (1976) Isolation and characterization of the cardiotonic polypeptide anthopleurin-A from the sea anemone *Anthopleura xanthogrammica*. *J. Pharm. Sc.* **65**, 1368-1374.

4. Tanaka, M., Haniu, K., Yasunobu, K. T. and Norton, T. R. (1977) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant, anthopleurin A. *Biochem.* **16**, 204-208.

5. Reimer, N. S., Yasunobu, C. L., Yasunobu, T. and Norton, T. R. (1985) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant anthopleurin-B. *J. Biol. Chem.* **260**, 8690-8695.

6. Bernheimer, A. W. and Avigad, L. S. (1982) Toxins of sea anemone *Epiactis prolifera*. *Arch. Biochem. Biophys.* **217**, 174-180.

7. Komatsu, K., Furukawa, K., Abe, K., Hirano, H. and Ueda, M. (1992) Isolation and characterization of equinatoxins from the sea anemone *Actinia equina* L. *Chem. Pharm. Bull.* **40**, 2873-2875.

8. Bernheimer, A. W. and Avigad, L. (1978) A cholesterol-inhibitable cytolytic protein from the sea anemone *Metridium senile*. *Biochim. Biophys. Acta* **541**, 96-106.

9. Rang, H. P. (1964) Stimulant actions of volatile anesthetics on smooth muscle. *Br. J. Pharmacol.* **27**, 256-375.

10. Hessinger, D. A. and Lenhoff, H. M. (1974) Degradation of red cell membrane phospholipids by sea anemone nematocyst venom. *Toxicon* **12**, 379-383.

11. Gillies, R. J., Dider, N. and Denton, M. (1986) Determination of cell number in monolayer cultures. *Anal. Biochem.* **159**, 109-113.

12. Barry, A. L. and Thornsberry, C. (1991) Susceptibility tests: Diffusion test procedures. In: *Manual of Clinical Microbiology.* (Balows, A., Hauser, W. J., Herrmann,

K. L., Isenberg, H. D. and Shadomy, H. J. eds.) pp. 1117-1152, American Society for Microbiology, Washington DC.

13. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

14. Spencer, R. L. and Wold, F. (1969) A new convenient method for estimation of total cystine-cysteine in proteins. *Anal. Biochem.* **32**, 191-197.

15. Huang, C. L. and Mir, G. N. (1972) Toxicological and pharmacological studies on sea anemone *Calliactis polypus* (Hormathiidae). *J. Pharm. Sc.* **61**, 66-69.

IV. SCREENING OF CRUDE EXTRACTS OF SEA ANEMONES FOR BIOLOGICAL ACTIVITY*

4.1 Introduction

For some time now, nematocysts, tentacles and whole bodies of sea anemones have provided metabolites possessing novel and/or known pharmacological and toxicological activities. Evidence of tissue differentiation into two layers, and organ formation within the animal kingdom, is first encountered in the Phylum Cnidaria. This characteristic arrangement of cells within the phylum suggests the existence of growth control substances preventing random cell growth.[1] In support of this, several researchers have shown that extracts of sea anemones from Hawaii and Tahiti[1-3] possess potent antitumor activity both *in vitro* and *in vivo*.

In 1974, while examining the extracts of different Cnidarians for potential antitumor activity, Shibata and his co-workers[4] found that the extracts caused *in vivo* cardiac stimulation in rats. For the first time, their report showed the presence of potential heart stimulants within the sea anemones.

Earlier studies had also demonstrated the existence of cytolytic (particularly hemolytic) activity in a number of sea anemones. Sea anemones native to the west coast

---

* A version of this chapter has been accepted for publication. Cline EI. and Wolowyk MW. **Int. J. Pharmacog.**

It was presented as a poster at the 34 th annual meeting of the American Society of Pharmacognosy, San Diego, CA July 18-22, 1993.

of United States have been the most widely investigated.[5] Since then, extracts of sea anemones from cold and warm waters have provided potent cytolytic agents.[84]

With this background, eleven sea anemones species common to the west coast of Canada were investigated for different biological activities. Extracts of different sea anemone species (Figure 4.1), seven of which had never before been investigated, were screened for cardiac stimulatory, cytotoxic, cytolytic and antifungal activities. This investigation was exploratory in nature and was carried out with a view to future in-depth study of the anemone species that showed most potent cardiac stimulatory action. It was also an attempt to search for new leads that would serve as potential cardiac stimulants of biochemical and pharmacological interests.

4.2 Results

4.2.1 Sea anemones

The sea anemones used in this research were identified by Seacology as already stated, and by the guidelines for identification provided by Kozloff.[6] Figure 4.1 shows some of the sea anemones as they appear within a tank of aerated sea water. Because of the small sizes and/or few numbers of some species, not all of them could be shown, since they had to be frozen on arrival. The eleven species used were *Anthopleura elegantissima*, *Anthopleura xanthogrammica*, *Corynactis californica*, *Epiactis prolifera*, *Metridium senile*, *Pachycerianthus fimbratus*, *Stomphia didemon*, *Urticina coriacea*, *U. crassicornis*, *U. lofotensis* and *U. piscivora*.

Figure 4.1 Appearance of sea anemones in a tank of aerated sea water. The different species are *Urticina lofotensis* (A), *U. piscivora* (B), *Anthopleura xanthogrammica* (C), *Epiactis prolifera* (D), *U. coriacea* (E), *U. crassicornis* (F), *Metridium senile* (G), *Stomphia didemon* (H) and *Pachycerianthus fimbratus* (I).

A
C
D
G

4.2.2 Extraction of sea anemones

All sea anemones were extracted as described (Section 3.2). The water soluble extracts obtained were stored at -70 °C and the yields obtained are indicated (Table 4.1). Powdered extracts constituted between 3 and 38 % of the total wet weight of the animals.

Table 4.1 Approximate yields of extracts of sea anemones

Sea anemone	Wet wt. (g)	Powdered extract (g)	Yield (%)
A. elegantissima (Brandt)	146	8.2	5.5
A. xanthogrammica (Brandt)	520	32.7	6.3
C. californica (Carlgren)	5.2	1.96	37.7
E. prolifera (Verrill)	72.1	10.8	15
M. senile L.	124.7	19.9	16
P. fimbratus	1005	67.8	6.7
S. didemon (Siebert)	250	21.6	8.6
U. coriacea (Cuvier)	1500	52.7	3.5
U. crassicornis (O. F. Muller)	441	37.4	8.5
U. lofotensis (Danielson)	860	37.4	4.3
U. piscivora (Sebens & Laakso)	1200	49.3	4.1

In parentheses are the taxonomists for each sea anemone. The taxonomist for *P. fimbratus* could not be established.

4.2.3 Cardiac stimulatory activity

Before the isolation and characterization process, several mammalian tissues were evaluated using the assay method already described, to determine which was most sensitive to the crude extracts. Three tissues were initially identified (rat and guinea pig left atria and guinea-pig ventricles). Rat and guinea-pig left atria proved to be very sensitive to the extracts while the ventricles did not. The former gave consistent positive inotropic activities while the ventricles gave very low and mostly negative inotropic responses as indicated in Figure 4.2. which illustrates a typical dose-response profile obtained from all three tissues. The rat atrium was chosen over guinea-pig because rats were much cheaper than guinea-pigs.

All the eleven extracts were assayed for cardiotonic activity using the left and right atria of rats. They all produced dose-dependent positive inotropic and chronotropic responses with the exception of *Pachycerianthus fimbratus*, which was inactive. While most extracts produced significant increases in heart rate, extracts of *Anthopleura xanthogrammica* and *Urticina crassicornis* produced the lowest change in heart rate (1.5 and 14.5 % increase respectively). Table 4.2 shows the maximum inotropic responses obtained for each sea anemone and the corresponding change in heart rate. Based on their EC_{50} values, the potencies of the extracts on the left atria were *A. elegantissima* > *A. xanthogrammica* > *U. piscivora* > *S. didemon* > *U. crassicornis* > *C. californica* > *U.*

lofotensis > *E. prolifera* > *U. coriacea* > *M. senile* > *P. fimbratus*. Tracings of the effect of *U. piscivora* on the left and right atria is illustrated (Figure 4.3).

There appeared to be no relationship between positive inotropic activity and the family to which the anemone belonged. For example, seven of the anemones assayed belonged to the family Actiniidae, but only four elicited maximum responses greater than 100 %. While the remaining species were lower, *U. coriacea* did not produce a 50 % response (Table 4.2). Such variations within the same family may be due to the presence of different classes and kinds of cardiotonic agents that may or may not act via the same mechanism(s). This can only be confirmed when the active agent(s) are purified and their mechanism of action evaluated.

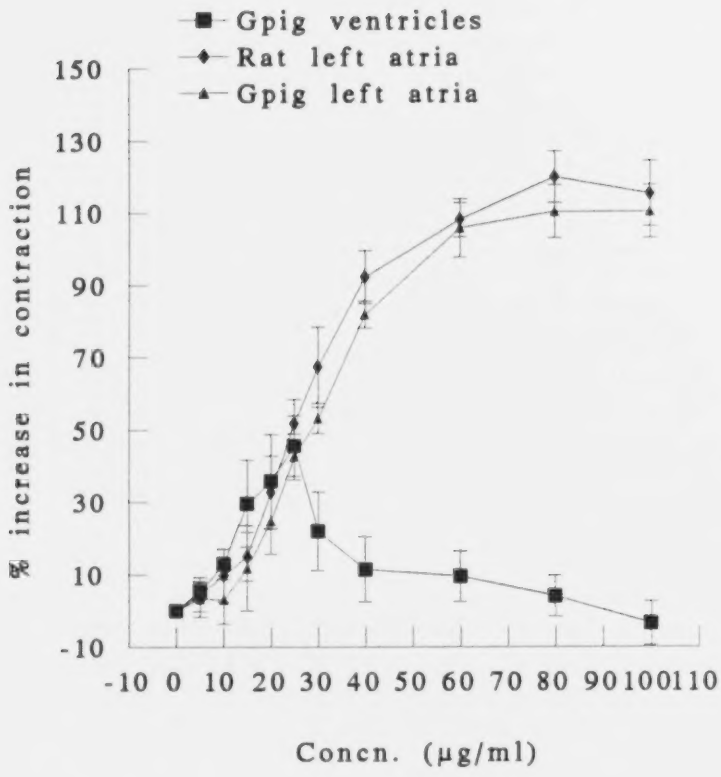


Figure 4.2 Typical profile of the effect of crude extracts of sea anemones (e.g. U. piscivora) on isolated tissues. Gpig (guinea-pig). Values are mean ± S. E. M. (n=3).

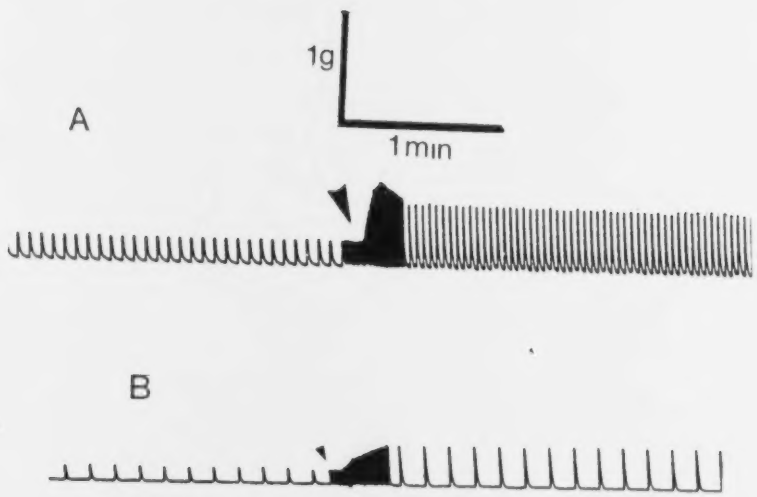


Figure 4.3 Tracings of the effect of crude extract (25 µg/ml) of *U. piscivora* on rat atrial tissues. (A) response from right atrium (chronotropy) and (B) response from left atrium (inotropy). Arrow indicates point of addition of extracts.

Table 4.2 Cardiotonic activity of crude extracts of sea anemones on rat atria

Sea anemone	EC_{50} (µg/ml)	Maximum response (%)	
		Inotropy	Chronotropy
A. elegantissima	5.0	164 ± 16.8	105 ± 7.6
A. xanthogrammica	5.1	102 ± 15.5	1.3 ± 3.1
C. californica	85	101 ± 13.4	62.9 ± 2.7
E. prolifera	72	74.7 ± 3.4	70 ± 30.2
M. senile	-	24.8 ± 11.4	32.2 ± 17.9
P. fimbratus	-	0	0
S. didemon	105	146.2 ± 36.3	47.9 ± 9.1
U. coriacea	-	33.2 ± 31.7	60.9 ± 32.2
U. crassicornis	98	102.5 ± 26.7	14.9 ± 9.3
U. lofotensis	105	80.8 ± 26.3	37.4 ± 7.6
U. piscivora	20	124.7 ± 8.7	72.4 ± 5.2

Values are mean ± S.E.M. (n = 3). Three rats were used for each assay. Increase in contraction and heart rate were noted for 5 min. until there was a decrease or no further change. (-) indicates 50 % contraction was not achieved. EC_{50} dose at which a 50 % response was obtained. (0) indicates inactive.

Of all the sea anemones assayed, only *E. prolifera* was cardiotoxic, giving rise to dysrhythmic responses. At higher doses, inotropic and chronotropic responses were completely inhibited. Figure 4.4 shows tracings of the cardiotoxic action produced by extracts of *E. prolifera*.

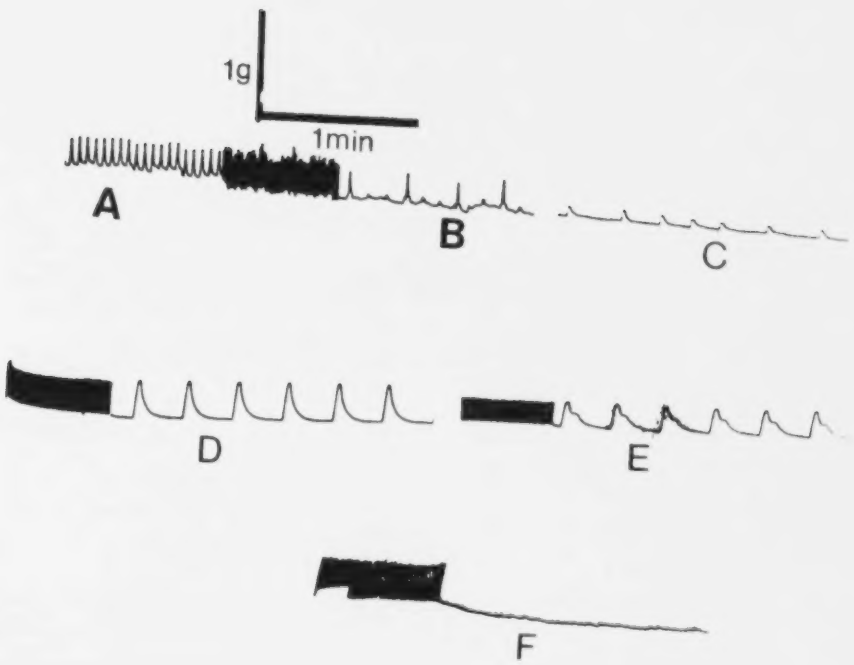


Figure 4.4 Tracings of the effect of crude extract (100 µg/ml) of *E. prolifera* on rat atrial tissues. (A) response of right atrium (RA) before addition of extract, (B) response of RA after addition of extract, (C) inhibition of chronotropic activity, (D) response from left atrium (LA) before addition of extract, (E) response of LA after addition of extract and (F) inhibition of inotropic activity.

4.2.4 Cytolytic activity of the extracts

Due to the unavailability of some of the powdered extracts, only extracts of eight anemone species (A. *xanthogrammica*, *M. senile*, *P. fimbratus*, *S. didemon*, *U. coriacea*, *U. crassicornis*, *U. lofotensis* and *U. piscivora*) were evaluated for hemolytic activity as described in Section 3.3.4. All extracts with the exception of *P. fimbratus* elicited potent lytic activity on three mammalian (rat, guinea-pig and dog) erythrocytes. The lytic action of the extracts are illustrated in Figures 4.5 - 4.7.

Rat erythrocytes appeared to be the most susceptible to lytic action by some extracts. However susceptibility of the erythrocytes to hemolysis varied with the anemone species. While rat erythrocytes appeared to be most susceptible to the lytic action of *U. piscivora*, they were less susceptible to extracts of *A. xanthogrammica*, *M. senile* and *U. lofotensis*.

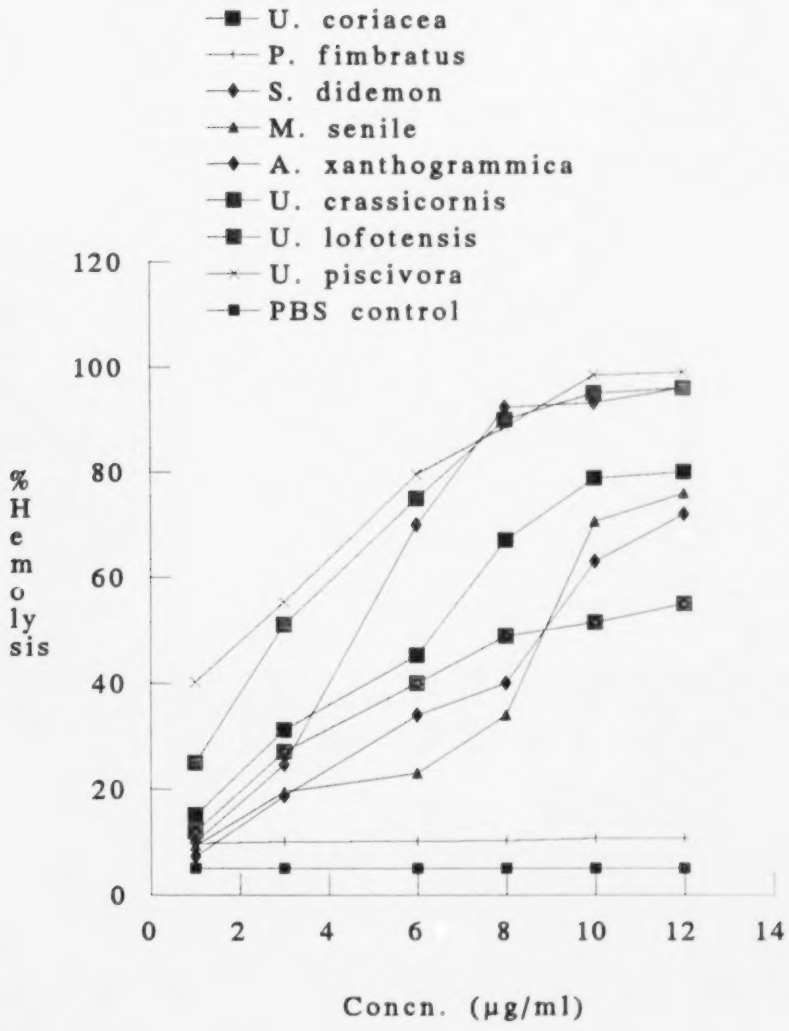


Figure 4.5 Relative cytolytic activity of sea anemone extracts to rat erythrocytes.

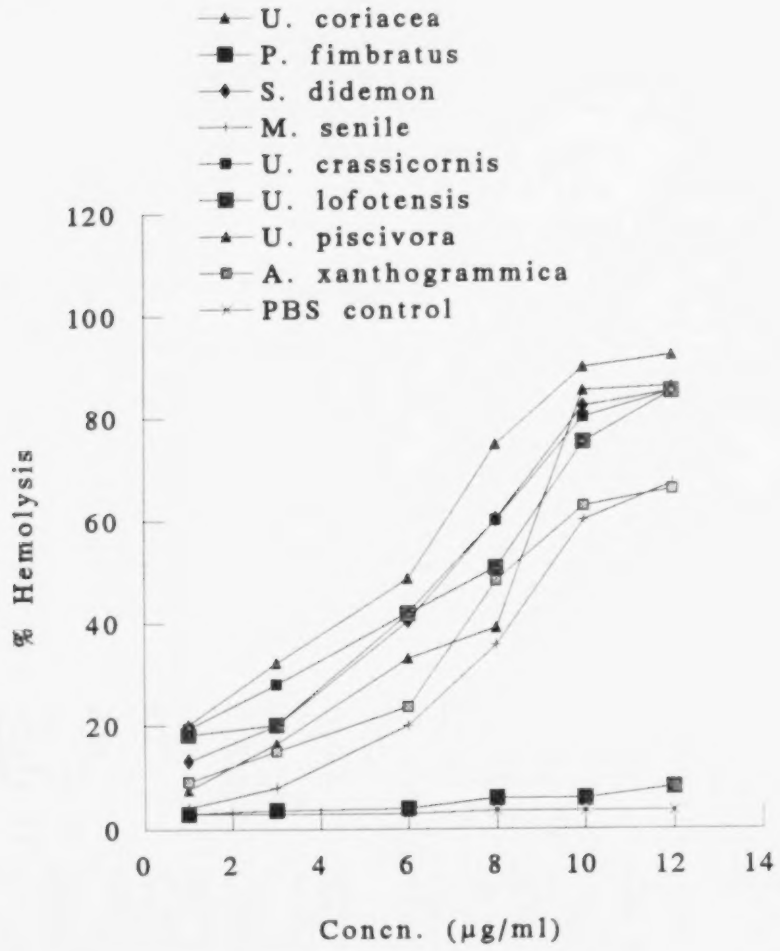


Figure 4.6 Relative cytolytic activity of sea anemone extracts to guinea pig erythrocytes.

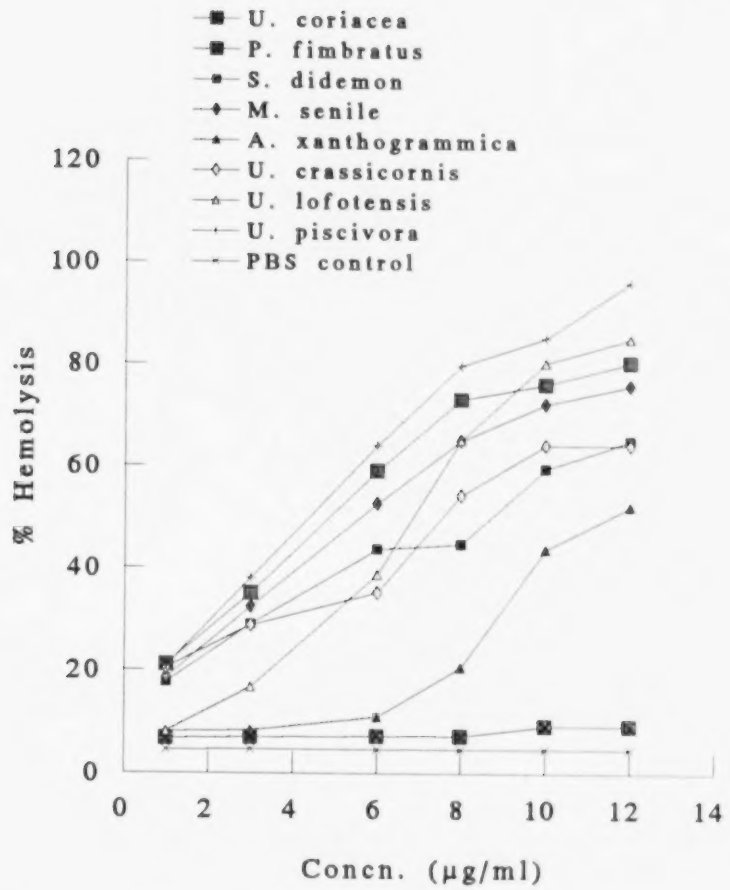


Figure 4.7 Relative cytolytic activity of sea anemone extracts to dog erythrocytes.

4.2.5 Cytotoxic activity of the extracts

Of the extracts evaluated for cytotoxic activity, *U. piscivora* and *U. coriacea* were the most potent, while the HEL 299 cell line appeared to be the most susceptible. The extracts were also toxic to KB and L1210 cells. Extracts of *A. xanthogrammica*, *S. didemon* and *U. lofotensis* were moderately cytotoxic. They were, however, more toxic to HEL 299 and L1210 cell lines than the KB cells. Extracts with toxicity values greater than 100 µg/ml for any particular cell line were considered non-toxic to those particular cells. Table 4.3 illustrates the TC_{50} values obtained for each extract assayed.

Table 4.3 Cytotoxic effect of crude extracts on three cell lines

Sea anemone	TC_{50} (µg/ml)		
	KB cell	L1210	HEL 299
A. xanthogrammica	100	97	50
P. fimbratus	> 100	97	55
S. didemon	100	50	25
U. coriacea	8	6	3
U. lofotensis	60	30	27
U. piscivora	6	10	2

TC_{50} concentration of crude extract (µg/ml) required to kill 50 % of cells within each cell line

4.2.6 Antifungal activity of the extracts

Extracts of all eleven species were assayed for antifungal activity against *Candida albicans* and *Aspergillus niger*. They were all inactive.

4.2.7 Protein determination

Extracts of all eleven species were evaluated for their protein content. Only extracts of ten sea anemone species gave positive reactions to the dye binding assay with varying degrees of protein content. *U. lofotensis* possessed the highest amount of protein per milligram of extract while *A. elegantissima* had the lowest. The extract of *P. fimbratus*, the only anemone devoid of cardiotonic and hemolytic activity, was completely devoid of protein. Since its extract showed moderate cytotoxic activity one is inclined to conclude that the active cardiotonic and hemolytic agent(s) may be protein in nature while the active cytotoxic agent(s) are not. The approximate protein content of each extract was interpolated from standard curves generated with (BSA) (Figure 4.8).

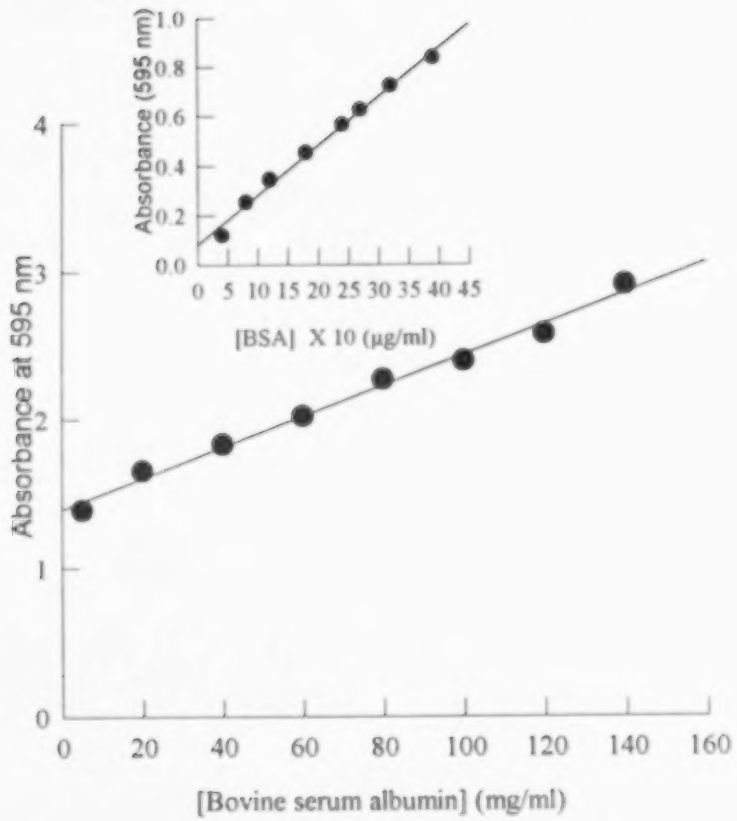


Figure 4.8 Standard curves generated for protein determination of crude extracts of sea anemones. Inset represents standard curve at lower (µg) concentrations. BSA- bovine serum albumin.

Table 4.4 Protein content (mg/ml) of crude extracts of sea anemones

Sea anemone	Total protein (mg/ml)
A. elegantissima	1.05
A. xanthogrammica	4.55
C. californica	7.89
E. prolifera	7.52
M. senile	69.6
P. fimbratus	0
S. didemon	38.8
U. coriacea	52.6
U. crassicornis	46.2
U. lofotensis	127.7
U. piscivora	18.2

(0) indicates lack of protein in crude extract.

4.3 Discussion and conclusions

The results reported show that water soluble extracts of sea anemones possess different biological activities, in this case cytotoxic, hemolytic and cardiotonic but not antifungal.

The method of extraction employed involved mechanical disruption in aqueous ethanol and this may have accounted for the presence of multiple biological activities.

Milder methods of extraction, such as freeze thaw cycles have resulted in lack of extraction of one group of compounds over the other.[7] This is due to localization of particular toxins within different parts of the anemone's body structure thus rendering them resistant to extraction.

The potent cardiotonic activity observed for *A. elegantissima* and *A. xanthogrammica* is consistent with the results of Shibata *et. al.*,[4] who first reported this activity. They, together with other researchers have since isolated and purified anthopleurin A, B and C (AP-A, AP-B, AP-C) from both species.[2,8,9]

Cytolytic activity utilizing mammalian erythrocytes has been widely investigated among sea anemones.[5,10] The active constituents have been shown to be polypeptides with molecular weights between 10-80 kDa.[11,12] Erythrocytes from some mammalian species have also been shown to be more sensitive to extracts of some anemone species than others. The susceptibility order for the crude extract of the sea anemone *Parascicyonis actinostoloides* was reported as cow > guinea pig > rabbit > horse > chicken.[13]

While there are many reports involving the hemolytic action of sea anemone extracts and purified toxins, there have been few on their cytotoxic activities. Those that have been assayed include extracts of *Stoichactis sp.* from Hawaii,[1] *Stoichactis kenti* from Tahiti[2] and A *elegantissima*.[3] They were all active against two experimental mouse tumors, P-388 lymphocyte leukemia and Ehrlich ascite tumor cells.

Mahnir *et al.*,[9] has since reported the absence of antifungal and antibacterial activity in 22 species of sea anemones from the North Pacific sea. The lack of

antimicrobial activity in their extracts and lack of antifungal activity in the extracts assayed here may be due to the concentration of the class of bioactive metabolite present in the tissue. This can be confirmed by screening pure metabolites for antifungal activity. However since the two fungal species assayed were not marine organisms, the negative result may reflect the response of sea anemones to local pathogenic invasion. The absence of these microorganisms used from the marine environment may be an indication that they do not pose any danger to the survival and or reproduction of sea anemones. However, it is still possible given the wide spectrum of bacteria and fungi that exists, that the extracts may be active on some other microorganisms. A second possibility is that the sea anemones assayed at present may lack antifungal activity, but others may exist that possess antimicrobial activity.

The results reported here were obtained from preliminary screening of the crude extracts, in an attempt to identify sea anemone species with potent biological activities particularly cardiotonic activity. The two most active species were *A. elegantissima* and *A. xanthogrammica*. Since the active constituents of these organism have been isolated and characterized[2-4] the next most potent anemone (*U. piscivora*) became the obvious candidate for further investigation. Subsequent chapters will highlight the methods used to isolate and characterize the active cardiotonic agent(s) from this organism and the evaluation of their biological activities.

Bibliography

1. Tabrah, F. L., Kashiwagi, M. and Norton, T. R. (1972) Antitumor activity in mice of four coelenterate extracts. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **5**, 420-422.

2. Norton, T. R., Kashiwagi, M., and Shibata, S. (1978) Anthopleurin A, B and C, cardiotonic polypeptides from the sea anemones *Anthopleura xanthogrammica* (Brandt) and *A. elegantissima* (Brandt). In: *Drugs and Food from the Sea.* (Kaul, P. N. and Sinderman, C. J. eds.) pp. 37-50, University of Oklahoma Press, Oklahoma.

3. Quinn, R. J., Kashiwagi, M., Norton, T. R., Shibata, S., Kuchi, M. and Moore, R. E. (1974) Antitumor activity and cardiac stimulatory effects of constituents of *Anthopleura elegantissima. J. Pharm. Sc.* **63**, 1798-1800.

4. Shibata, S., Dunn, D. F., Kuchi, M., Kashiwagi, M. and Norton, T. R. (1974) Cardiac stimulant action of extracts of coelenterates on rat atria. *J. Pharm. Sc.* **63**, 1332-1333.

5. Bernheimer, A. W. and Avigad, L. S. (1981) New cytolysins in sea anemones from the west coast of United States. *Toxicon* **19**, 529-534.

6. Kozloff, E. N. and Price, L. H. (1987) In: *Marine Invertebrates of the Pacific Northwest.* pp. 72-79, University of Washington Press, Seattle.

7. Kem, W. R. and Dunn, B. M. (1988) Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon* **26**, 997-1008.

8. Reimer, N. S., Yasunobu, C. L., Yasunobu, T. and Norton, T. R. (1985) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant anthopleurin-B. *J. Biol. Chem.* **260**, 8690-8695.

9. Tanaka, M., Haniu, K., Yasunobu, K. T. and Norton, T. R. (1977) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant, anthopleurin A. *Biochem.* **16**, 204-208.

10. Mahnir, V. M., Shumlov, U. N., Kovalevskaya, A. M., Romanenko, L. A. and Grebelny, S. D. (1993) Evidence for several types of biologically active substances in North Pacific sea anemones. *Comp. Biochem. Physiol.* **106C**, 661-665.

11. Macek, P. (1992) Polypeptide cytolytic toxins from sea anemones (Actinaria). *FEMS Microbiol. Immunol.* **105**, 121-130.

12. Turk, T. (1991) Cytolytic toxins from sea anemones. *J. of Toxicol.- Toxin Rev.* **10**, 223-262.

13. Shiomi, K., Tanaka, E., Yamanaka, H. and Kikuchi, T. (1985) Isolation and characterization of a lethal hemolysin in the sea anemone *Parasicyonis actinostoloides*. *Toxicon* **23**, 865-874.

V. ISOLATION AND CHARACTERIZATION OF THE CARDIOTONIC PROTEIN UPI FROM *URTICINA PISCIVORA*[*]

5.1 Introduction

Evidence for the presence of polypeptides and protein toxins within the nematocysts of sea anemones was first presented by Blanquet.[1] Since then considerable efforts have been devoted to the purification and characterization of these toxins.

Successful attempts to isolate and characterize these agents from crude extracts and or purified nematocysts did not come until the early seventies, when Hessinger and his co-workers[2] partially purified a phospholipase A-like toxin from the acontial nematocyst of the sea anemone *Aiptasia pallida*. They employed gel chromatography (Bio-Gel P-60) and three years later when gel filtration chromatography was replaced with anion exchange chromatography, four toxic components were identified.[3]

Since then, different chromatographic methods have been employed to isolate and characterize these toxins. Devlin[4] partially purified a toxin from the sea anemone *Stoichactis helianthus* from Barbados by first precipitating the crude extract with acetone. The resultant toxin was heterogeneous and could only be purified by preparative isoelectric focusing and was shown to have a molecular weight of 16 kDa.[5]

The most common methods employed today for successful purification of sea anemone toxins have been size exclusion chromatography, ion exchange chromatography with gradient elution using NaCl, and reverse phase HPLC.[6] For successful

---

[*] A version of this chapter has been published. Cline *et. al.*, 1995 **Pharm. Sc.** 1, 155-162.

It was presented as a poster at the 9th annual meeting of the American Association of Pharmaceutical Scientists, Nov. 6-10, 1994, San Diego CA.

characterization, researchers have made use of biochemical techniques such as SDS-PAGE, isoelectric focusing, amino acid analyses and N-terminal sequencing. All of these techniques together have provided information on the homogeneity, molecular weights and primary structure of the toxins. Despite these advances, the complete sequence of only two sea anemone toxins are known to date.[7,8]

The first cardiotonic polypeptide toxins from sea anemones, anthopleurin A, B and C (AP-A, B and C), from *Anthopleura elengantissima* and *A. xanthogrammica* sequenced by manual Edman degradation were shown to have molecular weights ~ 5 kDa.[9-11] Other than the anthopleurins, the only other cardiotonic peptide (tenebrosin A) to be sequenced was from the Australian sea anemone *Actinia tenebrosa*. It was characterized using a combination of gel filtration, reverse phase and cation exchange chromatography.[12]

Most high molecular weight toxins from sea anemones have been reported to be basic, consisting mainly of glycine, alanine, leucine, valine and threonine and lacking in cysteine residues. The most abundant aromatic residues have been tyrosine and phenylalanine.[8]

Despite these advances, there is still very little known regarding the structure and function of these molecules, especially the high molecular weight toxins. While the therapeutic potential of these polypeptides is yet to be realized,[13] they have demonstrated that sea anemones are useful sources of interesting cardiac stimulants. As a basis for the structural and functional characterization of cardiac stimulants within the sea anemone class, the isolation and characterization of one of the active cardiotonic agents from *Urticina piscivora* will be highlighted.

5.2 Results

5.2.1 Sea anemone

As already reiterated, *U. piscivora* was the anemone of choice because of the potent action it exhibited during preliminary screening.

5.2.2 Taxonomic classification of *U. piscivora* (Sebens & Laakso)

The classification employed was based on that of Friese.[14]

Kingdom - Animalia	Phylum - Cnidaria
Class - Anthozoa	Subclass - Hexacorallia
Order - Actinaria	Family - Actiniidae
Genus - *Urticina*	Species - *piscivora*

Figure 5.1 is a close up appearance of the sea anemone *Urticina piscivora* in a tank of aerated sea water.

5.2.3 Extraction

The extraction procedure utilized was the same as described in section 3.2. Wet drained *U. piscivora* (1.71 kg), was cut into small pieces and homogenized in 5.2 l 30 % (v/v) ethanol at 4 °C. From the lyophilized supernatant, a water soluble dry powder (103.4 g) was obtained constituting 6.8 % of the wet weight.

Figure 5.1 Close up appearance of *Urticina piscivora* (red column and white tentacles) in a tank of aerated sea water. Note the bulbous structures (cnidoblasts) at the tip of the tentacles, which house the nematocysts.

5.2.4 Purification of the active cardiotonic agent

The first step of purification was fractionation of the crude extract on Sephadex G-50 M. Separations were done in batches due to limitation in size of the column. Figure 5.2 is the chromatogram obtained from gel chromatography. Six fractions A (21-40), B (41-59), C (60-78), D (79-93), E (94-106) and F (107-130), were pooled, lyophilized and weighed. Each fraction was then assayed for cardiac stimulatory activity as described in section 3.3.2. The active fraction C (1.986 g) is the shaded region indicated in Figure 5.2 comprising a yield of about 1.9 % of the starting material.

The second step of purification on the other hand was needlessly complex as the active fraction C could not be retained on the available cation exchanger CM-cellulose, even at low pH's. Other ion exchangers employed were Mono S HR 5/5 cation and Mono Q HR 5/5 anion exchangers of the FPLC system. After several attempts, the active fraction was resolved on the cation exchanger SP-Sephadex C-25 at pH 4.5.

Fraction C (1.8 g) was then partially purified by cation exchange chromatography using an increasing concentration of ammonium acetate (10 mM - 1 M). Figure 5.3 is an illustration of the chromatogram obtained from this separation. Fractions were pooled, lyophilized, weighed and assayed for cardiac stimulatory activity. The active fraction (75 mg) is indicated by the shaded region corresponding to fractions 255-276. Isolation and characterization of proteins from the shaded region fractions 186-234 is described in the

next chapter. A schematic representation of the isolation and purification procedure is illustrated in Figure 5.11.

The lyophilized fraction (255-276) from the cation exchange separation (50 mg) was finally purified by a combination of preparative and analytical reverse phase HPLC as described in Section 3.4.3. Fractions from this separation were pooled, lyophilized, weighed and assayed to determine the active component. The active fraction, corresponding to a retention time of 23 min., was re-purified by analytical reverse phase HPLC to remove extraneous materials. It gave a pure active fraction (2.4 mg) which was designated UpI and which appeared homogenous. The chromatogram for UpI from analytical reverse phase HPLC is indicated in Figure 5.4. The insets (Figure 5.4 A and B) represent the first and second derivative UV spectra obtained by diode array detector. The former is a typical absorption spectrum for proteins and the latter indicates possible presence of aromatic amino acids. Lyophilized UpI was stored at -70 °C.

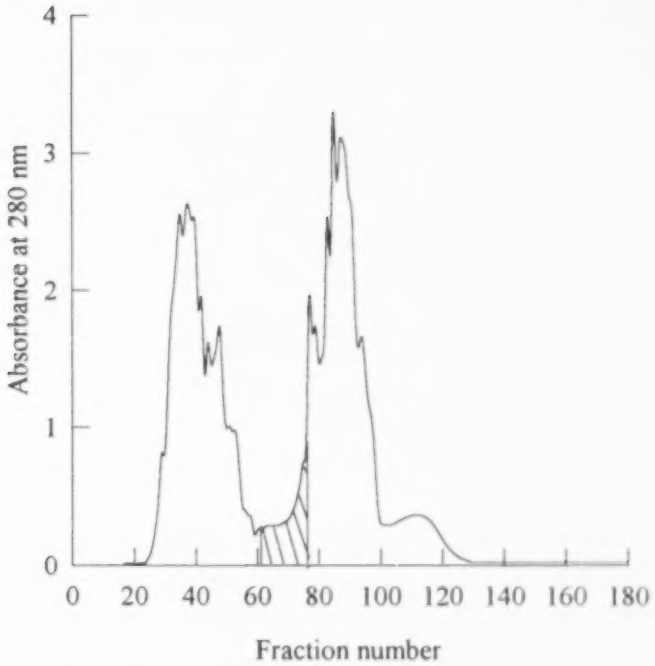


Figure 5.2 Gel chromatography on Sephadex G-50 M of lyophilized crude extract of *U. piscivora*. Eluting buffer is 0.1 M NH_4HCO_3, flow rate, 1 ml/min., at 4 °C. Shaded area indicates cardiac stimulatory activity. Pooled fractions are A (21-40), B (41-59), C (60-78), D (79-93), E (94-106) and F (107-130).

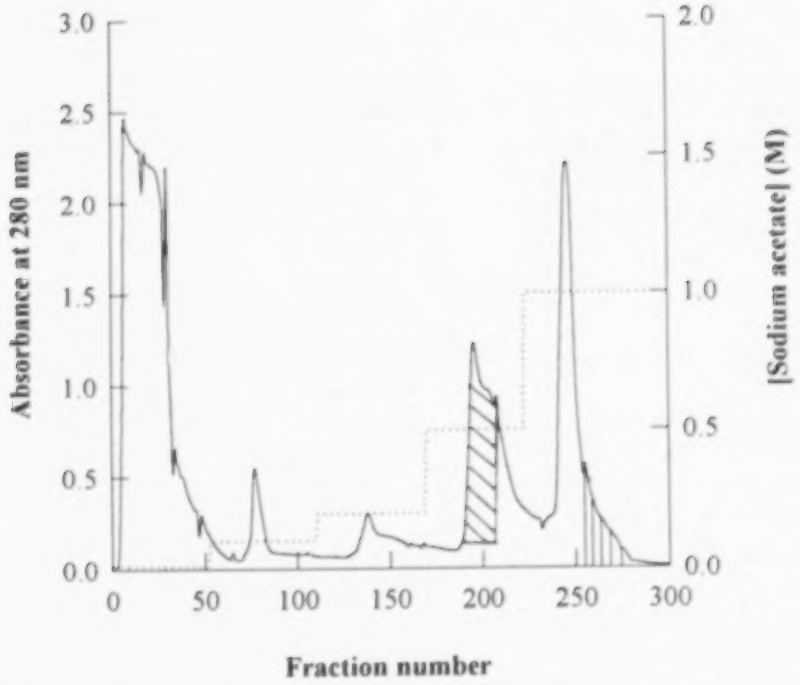


Figure 5.3 Ion exchange purification of active fraction C on SP-Sephadex C-25. Column size 23 x 3 cm, eluting buffer, sodium acetate pH 4.5, eluting gradient 10 mM NaAc 250 ml; 100 mM NaAc 200 ml; 200 mM NaAc 200 ml; 500 mM NaAc 200 ml and 1 M NaAc 300 ml. Flow rate 1 ml/min. Shaded areas indicate active cardiotonic fractions. UpI was purified from fraction 255-276 and UpII and UpIII from fraction 186-234 as described in the text.

5.2.5 Physicochemical characterization of UpI

UpI was further shown to be homogeneous from the following analyses: SDS-PAGE in both reducing and non-reducing conditions, isoelectric focusing, amino acid composition and N-terminal sequencing.

The molecular size of UpI was determined by SDS-PAGE as described in section 3.4.4.1. A calibration curve (Figure 5.5) was first generated using molecular markers described in section 3.4.4.1.1. Based on the distances migrated by UpI and the markers, the molecular weight of UpI was estimated as approximately 28 kDa (Figure 5.6 #2). The molecular weight was the same both in reducing and non-reducing conditions, indicating the absence of disulfide-linked subunits.

To determine the isoelectric point (pI) of UpI, a calibration curve (Figure 5.7) was first generated using pI markers as described in section 3.4.4.2. Based on the migration distances of the markers and UpI, its pI was determined to be > 9.3 (Figure 5.6 # 1). The actual pI could not be determined since the highest pI among the markers was 9.3.

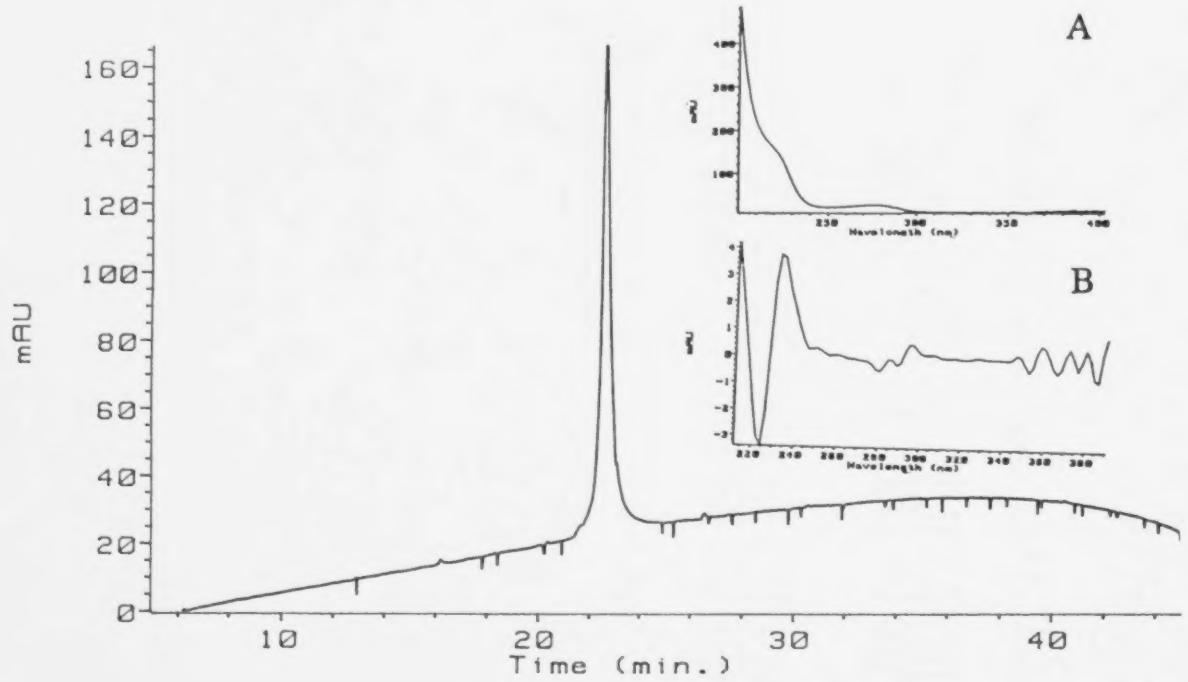


Figure 5.4 Analytical reverse-phase HPLC of purified UpI. A 30 µl sample in 0.05 % TFA (solvent A) was injected onto the column. The column was developed with a linear gradient from 0-70 % of 0.05 % TFA in acetonitrile pH 2 (solvent B) over a 35 min. period. Column: Zorbax 300 SB C_8, 15 x 2.1 mm, 300 °A, 5 µm particle size, flow rate 250 µl/min. at ambient temperature and 210 nm. The instrument consisted of a Hewlett Packard 1090 LC, fitted with a diode-array detector, an HP 85 computer, HP 7470A plotter and 79994A HPLC chemstation software. The absorption spectrum (A) and second derivative spectrum (B) is indicated by the inset.

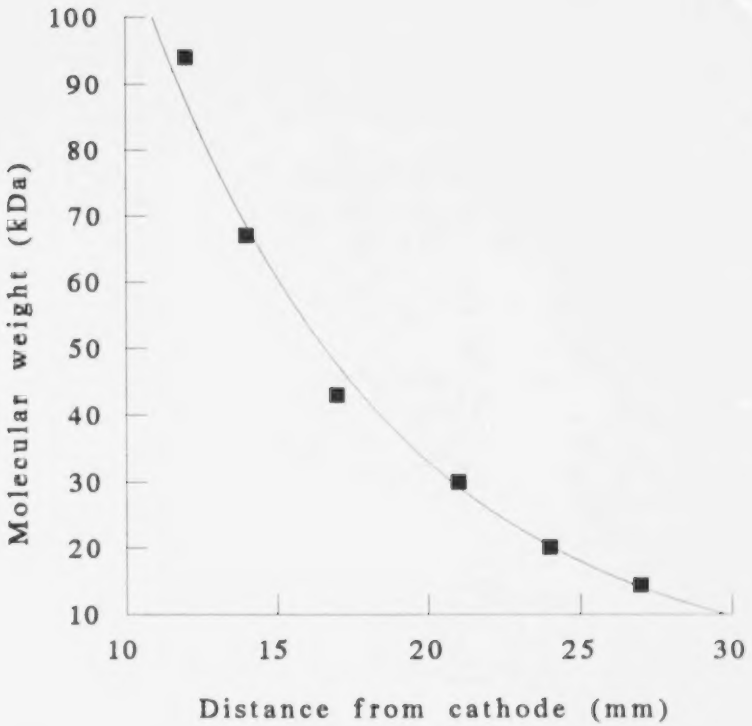
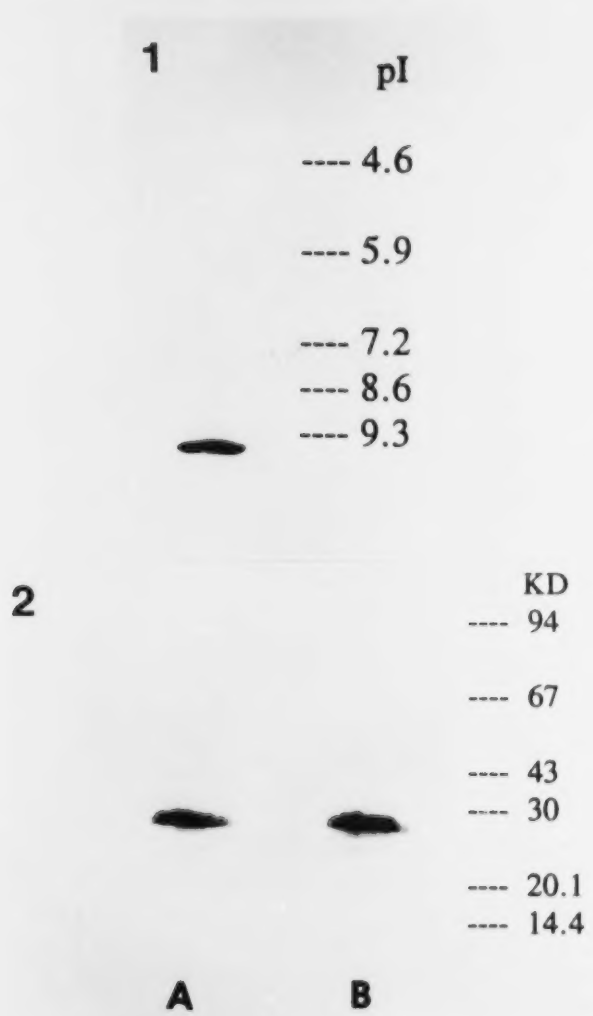


Figure 5.5 Calibration curve for molecular weight markers from phastGel SDS-PAGE. Markers are lactalbumin (14.4), trypsin inhibitor (20.1), carbonic anhydrase (30), ovalbumin (43), albumin (67), and phosphorylase b (94) kDa

Figure 5.6 Resolution of purified UpI by analytical isoelectric focusing (1) and SDS-PAGE (2).

1. 10 μg UpI was developed on IEF PhastGel 3-9 as described in text.

2. Electrophoresis was carried out in (A) presence of and (B) absence of β-mercaptoethanol as described in text. The pI and molecular weight markers are indicated on the respective sides of the gel lanes.


1
pI
---- 4.6
---- 5.9
---- 7.2
---- 8.6
---- 9.3
2
KD
---- 94
---- 67
---- 43
---- 30
---- 20.1
---- 14.4
A
B

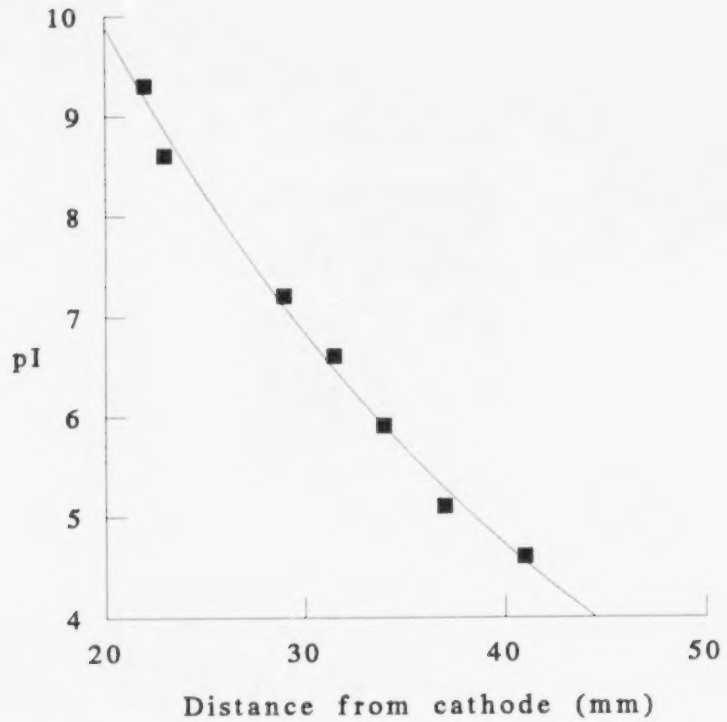


Figure 5.7 Calibration curve for pI markers from electrofocusing. Markers are trypsin inhibitor 4.6, myoglobin 7.2 ß-lactoglobulin 5.1, carbonic anhydrase I 5.9, carbonic anhydrase 6.6, L-lactic dehydrogenase 8.6 trypsinogen 9.3

5.2.5.1 Amino acid analysis of UpI

The amino acid composition of UpI is shown in Table 5.1. It revealed a molecular mass of 27.2 kDa which correlates with that obtained by SDS-PAGE. Tryptophan analysis was not carried out and this may or may not account for the slight difference in mass. UpI was rich in asparagine or aspartic acid (11.8 %), serine (8.2 %), glutamine or glutamic acid (7.8 %), glycine (10.2 %), alanine (8.6 %) and lysine (8.2 %). It was found to be poor in histidine and methionine residues (1.6 and 1.2 %) respectively. The aromatic amino acids tyrosine and phenylalanine and cysteine residues accounted for 3 % of the total residues respectively. The total number of amino acid residues was apparently 254, however this can only be determined accurately after elucidation of the complete primary structure.

5.2.5.2 N-terminal sequencing of UpI

Re-purified and intact UpI was subjected to automated Edman degradation as described in section 3.4.4.5. A total of 46 cycles was achieved after which subsequent products (phenylthiohydantoin or PTH-amino acids) could not be discerned. The first 46 amino acids are shown (Table 5.2). A search was carried out on the protein database Swiss-Prot[15] to determine if there were proteins with sequences similar to UpI. There was no similarity between UpI and other cytolysins previously isolated from sea anemone or

other organisms. However the search revealed some sequence similarity between UpI and the bungarotoxins from the snake venom of *Bungarus multicintus*. The extent of such similarity can only be determined from the complete primary structure of UpI.

Table 5.1 Amino acid composition of UpI

Amino Acid	Residue	Nearest Integer	Mole %
Asx	29.7	30	11.8
Thr	16.4	16	6.3
Ser	21.3	21	8.2
Glx	20.2	20	7.8
Gly	25.8	26	10.2
Ala	21.6	22	8.6
Cys	9.5	10	3.9
Val	9.5	10	3.9
Met	3.4	3	1.2
Ile	12.1	12	4.7
Leu	16.2	16	6.3
Tyr	10.1	10	3.9
Phe	8.3	8	3.2
Lys	21.4	21	8.2
His	3.9	4	1.6
Arg	9.5	10	3.9
Pro	15.4	15	5.9
Trp	nd	nd	nd
TOTAL		254	99.6
M_r		27,204	

nd not determined, Abbreviation: M_r molecular mass. Glx represents glutamine and or glutamic acid and Asx represents asparagine and or aspartic acid.

Table 5.2 Partial amino acid sequence of UpI showing the first forty-six residues.

UpI.	DENEN^{5}LYGPN^{10}ENKAK^{15}AKDLT^{20}AGASY^{25} LTKEA^{30}GCTKL^{35}QAGCT^{40}MYQAY^{45}N

5.2.3 Cardiac stimulatory activity of UpI

As already mentioned rat left atria were used as a bioassay during the separation procedure. It was also used to evaluate the dose response action of purified UpI for comparison with the known positive inotrope, isoproterenol (INA). A tracing of the effect of UpI on rat left atria is illustrated in Figure 5.8. UpI was active at concentrations as low as 10^{-12} M (calculations were made based on the molecular weight of 28 kDa). The dose response of UpI and INA is illustrated in Figure 5.9. UpI had an ED_{50} ($8.1x10^{-9}$ M), comparable to INA ($3x10^{-9}$ M). However INA was more efficacious than UpI as indicated in Figure 5.9 A. At higher concentrations (10^{-6} M), the response of the tissues to both substances was greatly reduced, indicating a probable desensitization of the tissues.

To confirm that UpI was actually the active cardiotonic agent, it was resolved on a 15 % gel by SDS-PAGE and its location on the gel cut off as strips. The gel strips which were extracted with Krebs-Hensleit buffer were then assayed for cardiac stimulatory activity as described in section 3.4.4.1.2. An activity profile of the strips obtained from the

gel is illustrated (Figure 5.10). Carditonic activity was limited only to the gel strip corresponding to UpI, thus indicating it was the active protein. A substantial loss in activity was observed (~70 %) and this was attributed to effects from the chemicals and reducing agents of electrophoresis.

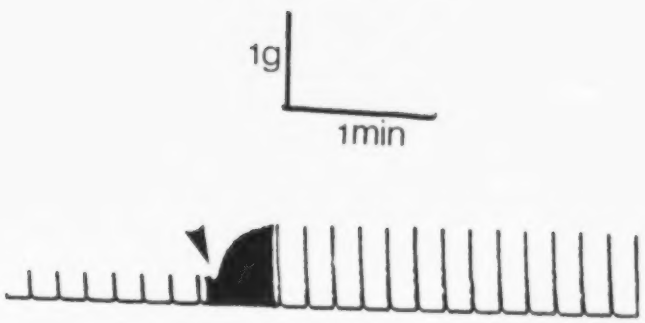


Figure 5.8 Tracing of the effect of UpI (10^{-7} M) on rat left atrium. Arrow indicates point of application of protein. Resting tension 1 g.

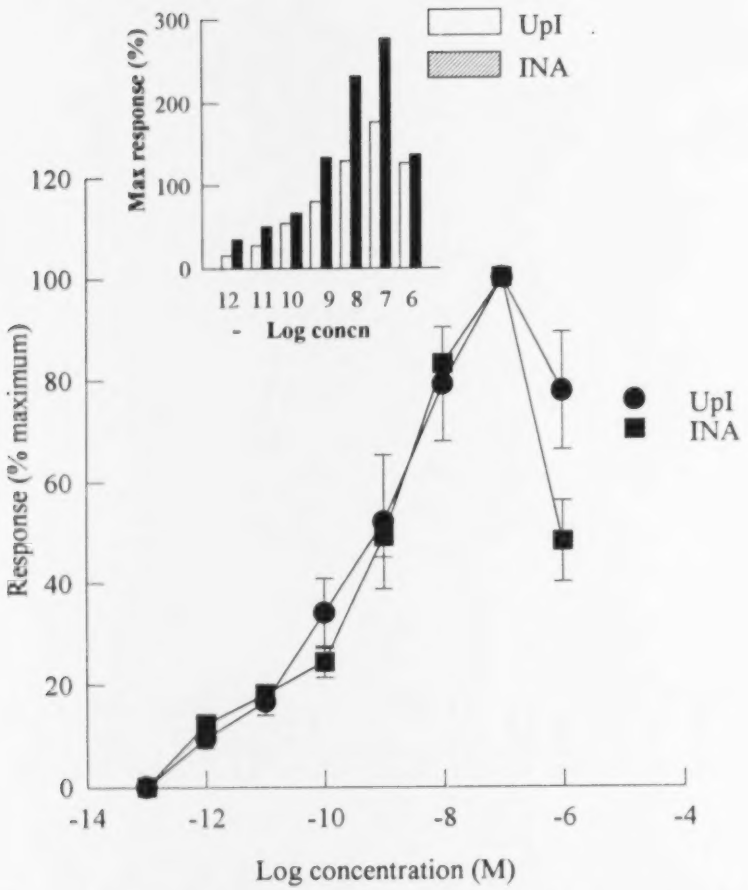


Figure 5.9 Dose response curves for UpI and isoproterenol (INA). Inset shows maximum responses for each of the concentrations tested on rat left atrial tissues. Values are mean ± S.E.M. (n=3).

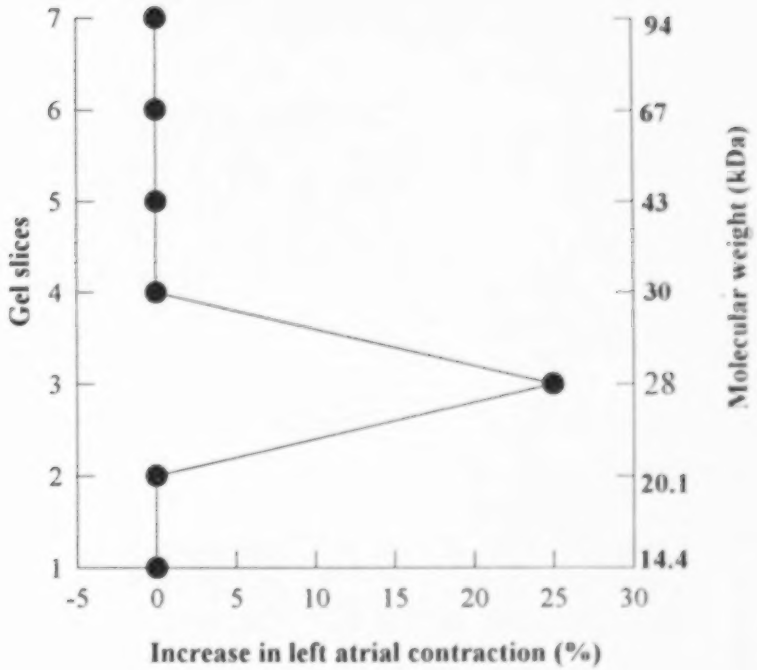


Figure 5.10 Plot of SDS-PAGE of a reverse phase HPLC purified UpI against positive inotropic activity. Resolving gel, 15 % under reducing conditions. Following electrophoresis, gels were sliced into strips corresponding to the molecular weight of standards and UpI (~ 28 kDa) and extracted as described in the text. The supernatant was assayed for cardiotonic activity. Activity was found only in the strip corresponding to UpI.

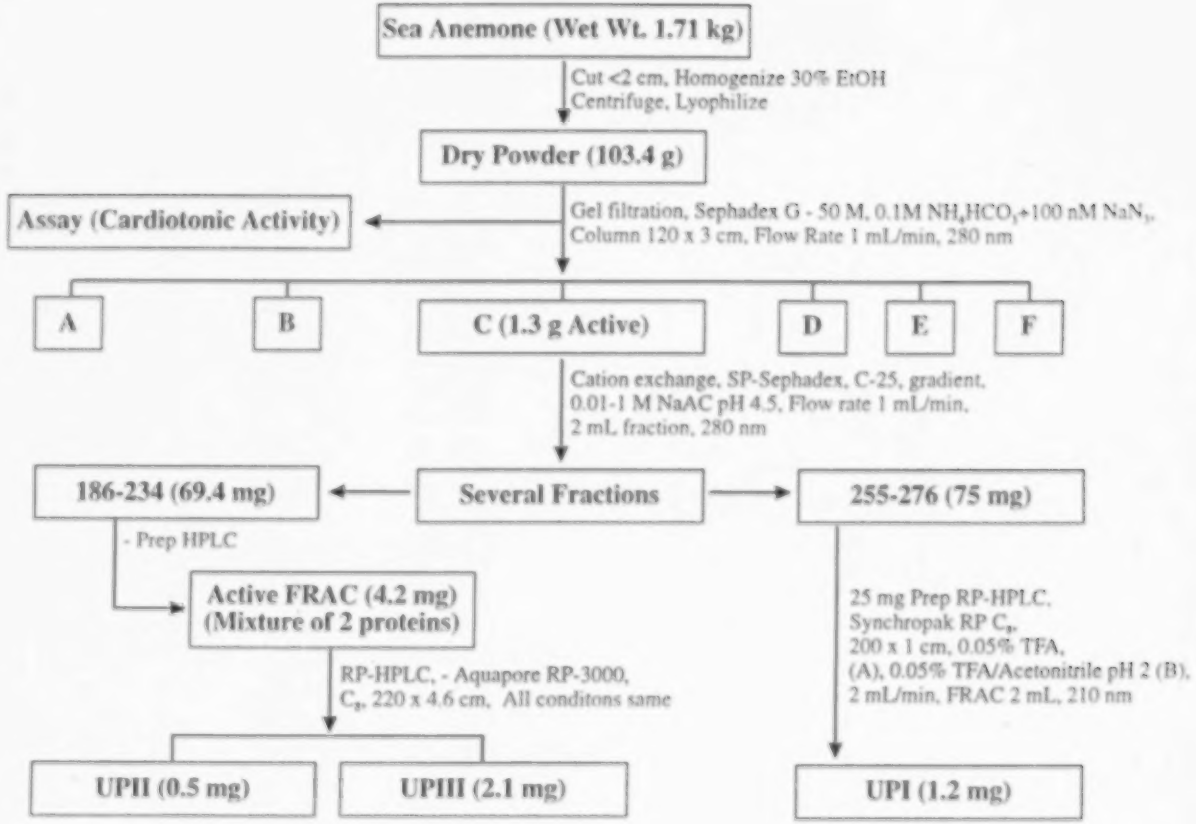

Sea Anemone (Wet Wt. 1.71 kg)
Cut <2 cm, Homogenize 30% EtOH
Centrifuge, Lyophilize
Dry Powder (103.4 g)
Assay (Cardiotonic Activity)
Gel filtration, Sephadex G - 50 M, 0.1M NH4HCO3+100 nM NaN3,
Column 120 x 3 cm, Flow Rate 1 mL/min, 280 nm
A
B
C (1.3 g Active)
D
E
F
Cation exchange, SP-Sephadex, C-25, gradient,
0.01-1 M NaAC pH 4.5, Flow rate 1 mL/min,
2 mL fraction, 280 nm
186-234 (69.4 mg)
Several Fractions
255-276 (75 mg)
- Prep HPLC
Active FRAC (4.2 mg)
(Mixture of 2 proteins)
RP-HPLC, - Aquapore RP-3000,
C8, 220 x 4.6 cm, All conditons same
25 mg Prep RP-HPLC,
Synchropak RP C8,
200 x 1 cm, 0.05% TFA,
(A), 0.05% TFA/Acetonitrile pH 2 (B),
2 mL/min, FRAC 2 mL, 210 nm
UPII (0.5 mg)
UPIII (2.1 mg)
UPI (1.2 mg)

Figure 5.11 Schematic representation of the isolation and purification of UpI-III as described in the text (Sections 3.4 - 3.4.3). Detailed description of the isolation and characterization of UpII and III is in Chapter 6.

5.2.4 Discussion and conclusions

The purification and preliminary characterization of a cardiac stimulatory protein from *U. piscivora* has been described. UpI is a basic protein consisting of ~ 254 amino acids, with an approximate molecular weight of 28 kDa.

The nomenclature (UpI) adopted was based on the first letters of the genus and species respectively and the number (I) indicates it was the first cardiotonic protein to be purified.

UpI was active on isolated rat atria at concentrations as low as 10^{-12} M, producing dose dependent responses. In this respect it was of similar potency to the sea anemone polypeptide AP-A from *A. xanthogrammica* and TN-A from *Actinia tenebrosa*. Both produce dose-dependent positive inotropic responses at concentration ranges of 2-8 nM[9,16] and 2 nM[12] respectively. A comparison of UpI with low molecular weight polypeptides (AP-A, B and C) is illustrated (Table 5.3). While there is a high degree of homology between the AP-A, B and C, there is very little similarity (only 3 similar residues) between the anthopleurins and UpI. This, together with the difference in molecular weight may be an indication that UpI belongs to a different class of sea anemone toxins.

The mechanism of cardiac stimulatory action of UpI was not determined but it is noteworthy that its molecular weight and partial sequence is different from the already known anemone cytolysins.[7] UpI becomes the second cytolysin other than TN-A to have

cardiotonic activity. Until now, potent cytolysins have been extracted from the venom of at least 19 species of sea anemones (Table 1.3) of which they constitute the most active constituent.[17,18] They have similar properties, are highly basic with pI's between 8 and 12 and are made up of single polypeptide chains. Many cytolysins have been isolated but only a few have been sequenced.[17] Among the nineteen cytolysins that are known to date, only two are different. They are phopsholipase-A_2 like toxins from *Aiptasia pallida*[19] and the cholesterol-inhibitable toxin (metridiolysin) from *Metridium senile*,[7] for which no sequence data are yet available.

Table 5.3 Comparison of N-terminal sequence of UpI from *U. piscivora* with the cardiotonic anthopleurins A, B and C from *A. elegantissima* and *A. xanthogrammica*

UpI	D	EN	E	N	LYGPNEN	KA	KAKDLTA	GA	S	YLTK	EAGC	TKL	QAGCTMYQ	A	YN
AP-A	.	GV	S		CLCDSDGP	SV	RGNTLSG	TL	W	LYPS	. . GC		PSGWHNCKAHG	D	TIGWCCLQ
AP-B	.	GV	P		CLCDSDGP	RP	RGNTLSG	IL	W	FYPS	. . GC		PSGWHNCKAHG	D	TIGWCCKK
AP-C	.	GV	P		CLCDSDGP	SV	RGNTLSG	IL	W	L . . .	AGC		PSGWHNCKAHG	P	TIGWCCKQ
				*			*				+	**			

Boxed letters indicates identical residues between the anthopleurins, (*) represents identical residues between UpI and all three anthopleurins, (+) represents identical residues between UpI and at least one anthopleurin. The references from which the sequences were obtained are (AP-A, Tanaka *et al.*[10]); (AP-B, Reimer *et al.*[11]) and (AP-C, Norton[13]). Gaps are introduced by program to give optimal alignment.

Considering the biochemical data presented for UpI, it can be concluded that it belongs to the third class of cytolysins (Section 1.3.2) reported to have phospholipase-A_2 action. UpI then becomes the first cytolysin within this class for which some sequence data is available. A comparison of UpI with other known cytolysins is illustrated in Table 5.4. It clearly shows that there is very little similarity between UpI and these known cytolysins. This clearly shows the uniqueness of sequence among the known sea anemone cytolysins and confirms its position within a different class.

Table 5.4 Comparison of N-terminal sequence of UpI from *U. piscivora* with cytolysins from sea anemones

UpI	DENENLYGPNE NKAK	A	K DLTA	G A	S Y L TK E A GCTKLQAGCTMY[1,2]
TN-A	NAAV	A	G AVI E	G A	T L T FE V L Q[1,2]
TN-B	SV AV	A	G AVI E	G A	T L T FN V L Q[2]
TN-C	SA DV	A	G AVI D	G A	S L S FD I L KTVLEALGNVKR[1]
EqTII	SA DV	A	G AVOD	G A	S L S FD I L K[1,3]
MagI	AL	A	G T I I A	G A	S L T FK I L DEV[2]
MagII	SA AL	A	G T I I D	G A	S L G FD I L NKV[2]
SH-I	S EL	A	G T I I D[2]		
SH-II	A EL	A	G T I I D[2]		
SH-III	AL	A	G T I I A	G A	SL T FQ V L DKV[2]
SH-IV	DQKEQTR AL	A	G T I I A[2]		
	* *		*	* *	*

Boxed letters indicate identical residues between UpI and other cytolyins. TN-A, B and C are variants from *Actinia tenebrosa* (Simpson *et al.*[23]); EqT II is equinatoxin from *Actinia equina* (Komatsu *et al.*[21]); SH-I to IV are variants from *Stichodactyla helianthus* (Kem and Dunn[6]) and Mag I & II are the variants magnificalysin I & II from *Heteractis magnifica* (Khoo *et al.*[20]). (*) indicates identical residue between UpI and at least one other cytolysin. Note the sequence similarity between all other cytolysins other than UpI. Gaps are introduced by program to give optimal alignment. 1. indicates positive inotropic activity; 2. hemolytic activity and 3. indicates negative inotropic activity.

As already stated, UpI showed some similarity to the bungarotoxins. A comparison of partial sequence of UpI with the bungarotoxins as obtained from Swiss-Prot is illustrated (Table 5.5).

Methods previously employed for the isolation of sea anemone cytolysins confirm that there is no uniform procedure for purification. Very few of these polypeptides and proteins have been obtained in sufficiently pure form to allow biochemical characterization.[7,28] The purification procedure described here resulted in a protein that was completely resolved, and the data presented establish beyond reasonable doubt that UpI is a basic protein with potent cardiotonic activity.

Table 5.5 Amino acid sequence homology among UpI from *U. piscivora* and the bungarotoxins from *Bungarus multicinctus*

```
pa20  MNPAHLLIL..SAVCVSLLGAANVPPQHLNLYQFKNMIVCAGT..RPWIG
pa21  ..............................NLINFMEMIRYTIPCEKTWGE
pa22  M....LIFLWCGAVCVSLLGAANIPPHPLNLINFMEMIRYTIPCEKTWGE
pa23  ..............................NLINFMEMIRYTIPCEKTWGE
pa24  MNPAHLLVL..SAVCVSLLGAANIPPQHLDLYQFKEMIRYTIPCEKTWIG

 UpI  ........................DENE N L YG PNENKAKAKDL T AGA SY .
pa20  YVNYGCYCGAGGSGTPVDELDRCCYVHD N C YG EAEKIPGCNPK T KTY SY T
pa21  YADYGCYCGAGGSGRPIDALDRCCYVHD N C YG DAEKKHKCNPK T SQY SY K
pa22  YADYGCYCGAGGSGRPUDAKDRCCYVKD N C YG DAEKKHKCNPK T QSY SY K
pa23  YTDYGCYCGAGGSGRPIDALDRCCYVKD N C YG DDAAIRDCNPK T SQY SY K
pa24  YADYGCYCGAGGSGTPIDALDRCCYVKD N C YG DAANIRDCDPK T QSY SY K
                                          ***

 UpI  L TK EA..............G C TKLQ A G C TMYQA Y N
pa20  C TK PNLTCTDAAGTCARIVCD C DRTA A L C FAAAP Y NINNFMISSSTHCQ
pa21  L TK RTIICYGAAGRGCRIVCD C DRTA A L C FGQSD Y IEGHKNIDTARFCQ
pa22  L TK RTIICYGAAGTCARIVCD C DRTA A L C FGNSE Y IERHKNIDTKRHCR
pa23  L TK RTIICYGAAGTCARVVCD C DRTA A L C FGQSD Y IEGHKNIDTARFCQ
pa24  L TK RTIICYGAAGTCARVVCD C DRTA A L C FGNSE Y IEGHKNIDTARFCQ
      *                                          *
```

pa20, non toxic phospholipase A_2 (Danse,[24]); pa21, ß-bungarotoxin A_1 chain (Danse *et al.*[25]); pa22, ß-bungarotoxin A_2 chain precusor (Danse *et al.*[26]); pa23, ß-bungarotoxin A_3 chain (Kondo *et al.*[27]); pa24, ß-bungarotoxin A_4 chain (Kondo *et al.*[27]). Boxed letters represent identical residues across the sequence; (*) represents identical residues between UpI and at least one other sequence.

Bibliography

1. Blanquet, R. (1968) Properties and composition of the nematocyst toxin of the sea anemone *Aiptasia pallida*. *Comp. Biochem. Physiol.* **25**, 893-902.

2. Hessinger, D. A., Lenhoff, H. M. and Kahan, L. B. (1973) Hemolytic, phospholipase A and nerve-affecting activities of sea anemone nematocyst venom. *Nature-New Biol.* **241**, 125-127.

3. Hessinger, D. A. and Lenhoff, H. M. (1976) Membrane structure and function: Mechanism of hemolysis induced by nematocyst venom-Role of phospholipase A and direct lytic factor. *Arch. Biochem. Biophys.* **173**, 603-613.

4. Devlin, J. P. (1974) Isolation and partial purification of hemolytic toxin from sea anemone, *Stoichactis helianthus*. *J. Pharm. Sc.* **63**, 1478-1480.

5. Bernheimer, A. W. and Avigad, L. (1976) Properties of a toxin from the sea anemone *Stoichactis helianthus*, including specific binding to sphingomyelin. *Proc. Nat. Acad. Sc. USA* **73**, 467-471.

6. Kem, W. R. and Dunn, B. M. (1988) Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon* **26**, 997-1008.

7. Macek, P. (1992) Polypeptide cytolytic toxins from sea anemones (Actinaria). *FEMS Microbiol. Immunol.* **105**, 121-130.

8. Turk, T. (1991) Cytolytic toxins from sea anemones. *J. Toxicol.-Toxin Rev.* **10**, 223-262.

9. Norton, T. R., Shibata, S., Kashiwagi, M. and Bentley, J. (1976) Isolation and characterization of the cardiotonic polypeptide anthopleurin-A from the sea anemone *Anthopleura xanthogrammica*. *J. Pharm. Sc.* **65**, 1368-1374.

10. Reimer, N. S., Yasunobu, C. L., Yasunobu, T. and Norton, T. R. (1985) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant anthopleurin-B. *J. Biol. Chem.* **260**, 8690-8693.

11. Tanaka, M., Haniu, K., Yasunobu, K. T. and Norton, T. R. (1977) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant, anthopleurin-A. *Biochem.* **16**, 204-208.

12. Thomson, M., Moritz, R. L., Simpson, R. J. and Norton, R. S. (1987) Tenebrosin-A, a new cardiostimulant protein from the Australian sea anemone *Actinia tenebrosa*. *Biochem. Int.* **15**, 711-718.

13. Norton, T. R. (1981) Cardiotonic polypeptides from *Anthopleura xanthogrammica* (Brandt) and *A. elegantissima* (Brandt). *Fed. Proc.* **40**, 21-25.

14. Friese, U. E. (1972) *Sea Anemones.* pp. 1-128, T F. H. Publications Inc. Ltd. Hong Kong.

15. Bairoch, A. and Boeckman, B. (1992) The Swiss-Prot protein sequence data bank. *Nucleic Acids Res.* **20**, 2019-2022.

16. Shibata, S., Norton, T. R., Izumi, T. Matsuo, T. and Katsuki, S. (1976) A polypeptide (AP-A) from sea anemone (*Anthopleura xanthogrammica*) with potent positive inotropic action. *J. Pharmacol. Exp. Ther.* **199**, 298-309.

17. Harvey, H. L. (1990) Cytolytic toxins. In: *Handbook of Toxinology* (Shier, W. T. and Mebs, D. eds.) pp. 1-66, Marcel Dekker, New York.

18. Kem, W. R. (1988) Sea anemone toxins: structure and action. In: *Biology of Nematocysts.* (Hessinger, D. A. and Lenhoff, H. M. eds.) pp. 375-405, Academic Press Inc. New York.

19. Hessinger, D. A. and Lenhoff, H. M. (1973) Assay and properties of the hemolysis activity of pure venom from the nematocysts of the acontia of the sea anemone *Aiptasia pallida*. *Arch. Biochem. Biophys.* **159**, 629-638.

20. Khoo, K. S., Kam, W. K., Khoo, H. E., Gopalakrishnakone, P. and Chung, M. C. M. (1993) Purification and partial characterization of two cytolysins from a tropical sea anemone *Heteractis magnifica*. *Toxicon* **31**, 1567-1579.

21. Komatsu, K., Furukawa, K., Abe, K., Hirano, H. and Ueda, M. (1992) Isolation and characterization of equinatoxins from the sea anemone *Actinia equina* L. *Chem. Pharm. Bull.* **40**, 2873-2875.

22. Mebs, D., Claus, I., Schroter, A., Takeya, H., and Iwanga, S. (1992) Hemolytic proteins from sea anemones. In: *Recent Advances in Toxinology Research.* (Gopalakrishnakone, P. and Tan, C. K. eds.) pp. 392-395, National University of Singapore, Singapore.

23. Simpson, R. J., Reid, G. E., Moritz, R. L., Morton, C. and Norton, R. S. (1990) Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and hemolytic protein from the sea anemone *Actinia tenebrosa*. *Eur. J. Biochem.* **190**, 319-328.

24. Danse, J. (1990) Nucleotide sequence encoding for non-toxic phospholipase A_2 from *Bungarus multicintus*. *Nucleic Acids Res.* **18**, 4608.

25. Danse, J., Garnier, J. and Kempf, J. (1990) cDNA deduced amino-acid sequence of a new phospholipase from *Bungarus multicintus*. *Nucleic Acids Res.* **18**, 4610.

26. Danse, J., Toussaint, J. and Kempf, J. (1990) Nucleotide sequence encoding β-bungarotoxin A_2-chain from the venom glands of *Bungarus multicintus*. *Nucleic Acids Res.* **18**, 4609.

27. Kondo, K., Toda, H., Narita, K. and Lee, C. (1982) Amino acid sequences of three β-bungarotoxins (β_3, β_4 and β_5-bungarotoxins) from *Bungarus multicintus* venom. Amino acid substitutions in the A chains. *J. Biochem.* **91**, 1531-1548.

28. Macek, P., Sencic, L. and Lebez, D. (1982) Isolation and partial characterization of three lethal and hemolytic toxins from the sea anemone *Actinia cari*. *Toxicon* **20**, 181-185.

VI. SEPARATION AND CHARACTERIZATION OF CARDIAC STIMULATORY PROTEIN VARIANTS UPII AND UPIII FROM *URTICINA PISCIVORA*[*]

6.1 Introduction

Among the polypeptide toxins found in sea anemones, be it from the tentacle or whole body extracts, are a group of closely related toxins described as protein variants, isoforms or isotoxins. To date all known isotoxins are cytolytic and show a high degree of homology within individual species.[1-3]

Kem and Dunn,[4] first reported the presence of four closely related cytolysins (SH I-IV) from the sea anemone *Stichodactyla helianthus*. Their molecular weights from SDS-PAGE analysis ranged from 17 to 20 kDa. Since then isotoxins as called isoform or variants have been reported in five different species of sea anemones.[3]

From the exudate of tentacles of *Actinia equina*, Macek and Lebez[5] separated three isotoxins designated equinatoxin I, II and III (EqT I - III), with similar molecular weights of 18 kDa. Using a modified isolation method of Ferlan and Lebez[6] and a combination of chromatographic methods, Komatsu *et al.*[7] reported the equinatoxins as hemolytic glycoproteins with molecular weights of 20 kDa.

---

[*] A version of this chapter has been accepted for publication. Cline *et al.*, **Int. J. Bio-Chromatog.** (In Press).

It was presented as a poster at the 36th annual meeting of the American Society of Pharmacognosy, University of Mississippi, July 23-27 1995.

In another study, crude extracts obtained from whole bodies of *Epiactis prolifera* were separated by means of preparative electrofocusing to yield three isotoxins named epiactin A, B and C. They all lacked methionine residues.[8]

In 1982 Macek and his co-workers[9] reported the presence of three closely related toxins in extracts of *Actinia cari*. Using a combination of gel, ion exchange chromatography and disc-PAGE, they isolated caritoxin I, II and III (CTX I-III) with estimated molecular weights of 20-25 kDa. However using a novel method of isolation called "milking", (venoms from sea anemones are obtained by pressing individual specimens over a beaker over a long period of time), Sencic and Macek[10] were able to separate two toxins (CTX I and II) from the same species and established their molecular weights as 19.8 kDa. Their study clearly suggested that there were differences between crude venom obtained from whole bodies and those from the tentacles, as they could not isolate CTX III.

Norton *et al.*,[11] extracted and isolated three isotoxins from *Actinia tenebrosa* designated tenebrosin A, B and C (TN-A, B and C). They showed for the first time the existence of cardiotonic activity among the isotoxins. The variants of the tenebrosin are basic proteins with different molecular weights (19-20 kDa). TN-C has since been reported to be very similar to EqT II.[12]

The most recent of the cytolytic isotoxins to be isolated was from the sea anemone *Heteractis magnifica*.[13] They were potent hemolytic proteins designated magnificalysin I and II with similar molecular weights of 19 kDa.

In the continued search for cardiac stimulatory agents from *Urticina piscivora*, two active proteins were isolated that appeared to be closely related biochemically. They have been designated UpII and UpIII and their methods of separation and characterization are highlighted in this Chapter.

6.2 Results

6.2.1 Sea anemone

U. piscivora was the anemone of choice for reasons already stated and the extraction procedure is as described in Section 3.2.

6.2.2 Separation and purification of cardiotonic variants

The first step in the purification of the isotoxins from the crude extract of *U. piscivora* was gel chromatography on Sephadex G-50 M as described in Section 3.4.1. Separations were carried out in batches and the chromatogram obtained is the same from which UpI was isolated (Figure 5.2). The active cardiotonic fraction C is shaded.

Active fraction C (1.8 g) was then separated by cation exchange chromatography, SP-Sephadex C-25, using an increasing concentration of sodium acetate (10 mM-1 M) pH 4.5. Fractions were pooled, lyophilized and assayed for cardiac stimulatory activity. The chromatogram obtained is illustrated in Figure 5.3 and the active fraction (64.9 mg) corresponding to fractions (186-234) is indicated by the shaded area.

Analysis of this active fraction by analytical reverse phase HPLC, fitted with a diode array detector revealed the presence of several protein peaks, (Figure 6.1) which could only be discerned as a single band by SDS-PAGE.

Separations of the two peaks (indicated by the arrows) was achieved by use of preparative reverse phase HPLC. The active fraction (25 mg) was first chromatographed on a reverse phase column as described in Section 3.4.3. Fractions were collected, assayed and lyophilized to give an active fraction (4.2 mg). This fraction, however, was still heterogeneous and consisted of both proteins, which were finally separated by re-chromatography on a smaller reverse phase column (Section 3.4.3). Two active purified protein fractions were obtained designated UpII (0.5 mg) and UpIII (2.1 mg). Analytical reverse phase HPLC of both proteins (Figures 6.2 and 6.3) confirmed closely related retention times of 17.1 and 17.8 min. respectively. A schematic representation of the isolation and purification procedure is illustrated in Figure 5.11.

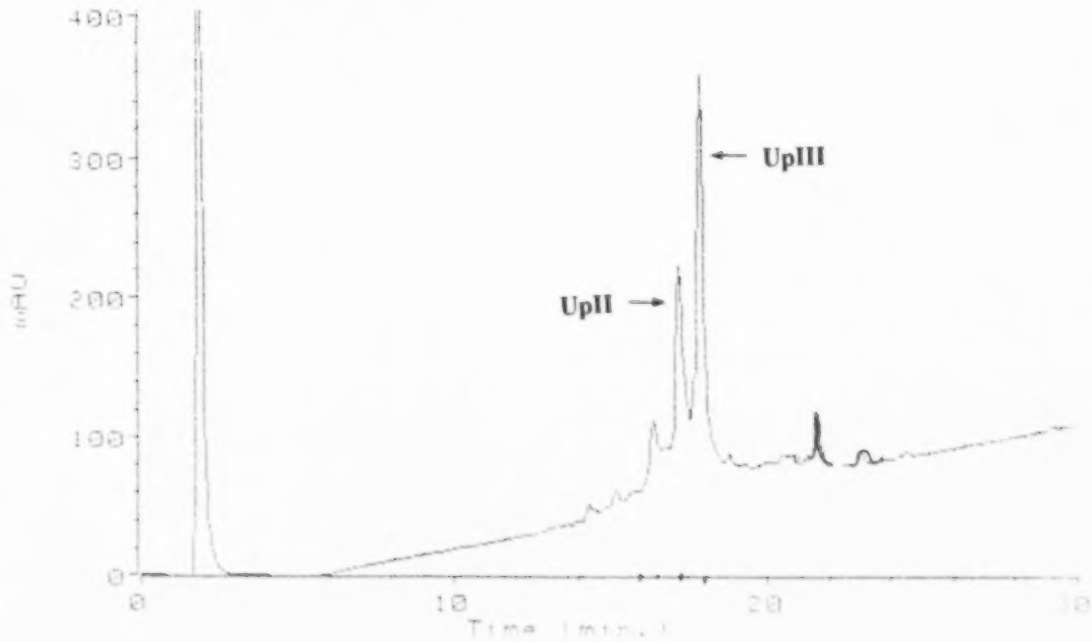


Figure 6.1 Analytical reverse-phase HPLC of pooled fraction obtained by preparative reverse phase chromatography. A 30 µl sample in 0.05 % TFA (solvent A) was injected onto the column. The column was developed with a linear gradient from 0-70 % of 0.05 % TFA in acetonitrile pH 2 (solvent B) over a 20 min. period. Column: Zorbax 300 SB C_8, 15 x 2.1 mm, 300 °A, 5 µm particle size, flow rate 250 µl/min. at ambient temperature and 210 nm. The instrument consisted of a Hewlett Packard 1090 LC, fitted with a diode-array detector, an HP 85 computer, HP 7470A plotter and 79994A HPLC chemstation software. UpII and UpIII were eventually purified from this fraction as described in the text. Arrows indicates positions of UpII and UpIII respectively.

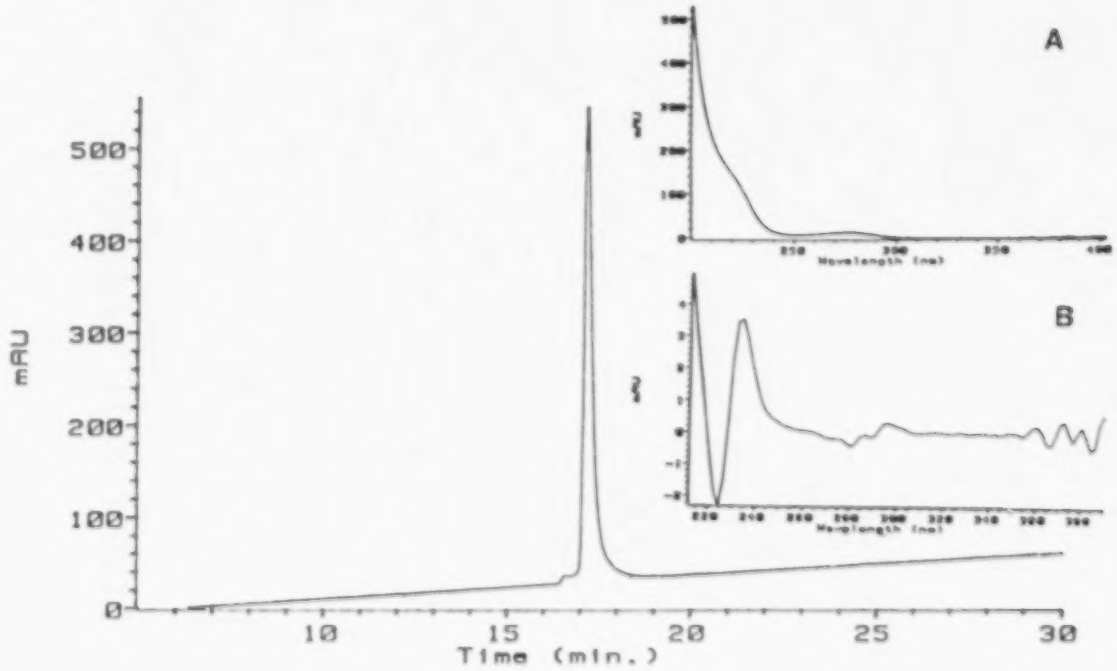


Figure 6.2 Analytical reverse-phase HPLC of purified UpH. A 30 µl sample in 0.05 % TFA (solvent A) was injected onto the column. The column was developed with a linear gradient from 0-70 % of 0.05 % TFA in acetonitrile pH 2 (solvent B) over a 35 min. period. Column: Zorbax 300 SB C_8, 15 x 2.1 mm, 300 °A, 5 µm particle size, flow rate 250 µl/min. at ambient temperature and 210 nm. The instrument consisted of a Hewlett Packard 1090 LC, fitted with a diode-array detector, an HP 85 computer, HP 7470A plotter and 79994A HPLC chemstation software. The absorption spectrum (A) and second derivative spectrum (B) is indicated by the inset.

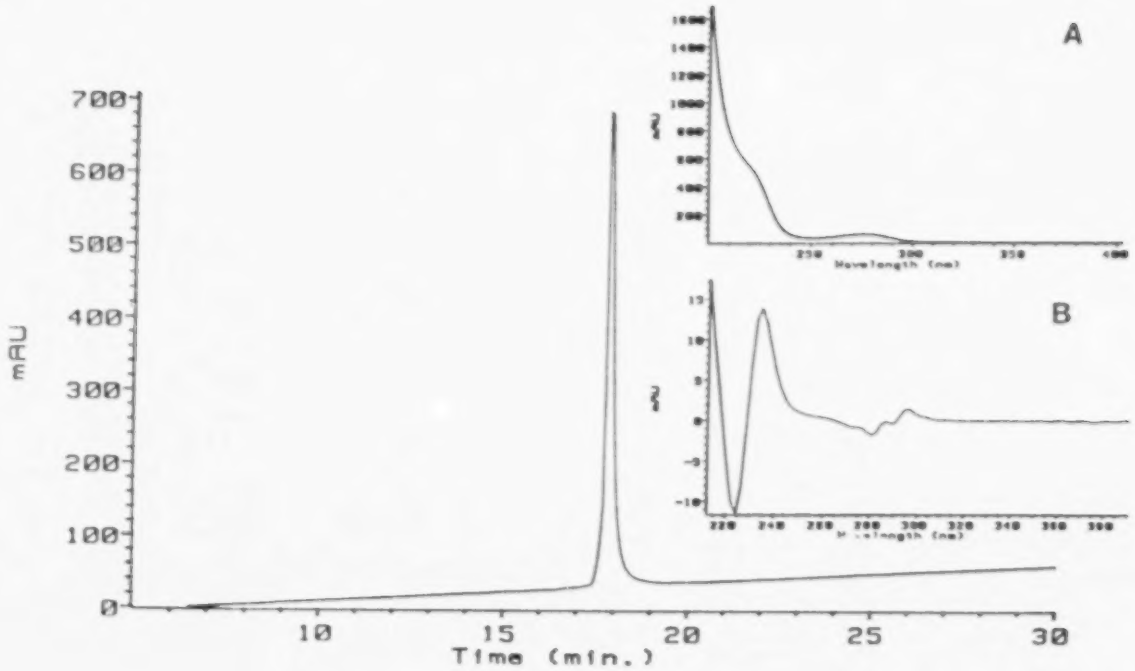


Figure 6.3 Analytical reverse-phase HPLC of purified UpIII. A 30 µl sample in 0.05 % TFA (solvent A) was injected onto the column. The column was developed with a linear gradient from 0-70 % of 0.05 % TFA in acetonitrile pH 2 (solvent B) over a 35 min. period. Column: Zorbax 300 SB C_8, 15 x 2.1 mm, 300 °A, 5 µm particle size, flow rate 250 µl/min. at ambient temperature and 210 nm. The instrument consisted of a Hewlett Packard 1090 LC, fitted with a diode-array detector, an HP 85 computer, HP 7470A plotter and 79994A HPLC chemstation software. The absorption spectrum (A) and second derivative spectrum (B) is indicated by the inset.

6.2.3 Physicochemical characterization of protein variants

Both polypeptides were shown to be homogenous by analytical reverse phase HPLC, SDS-PAGE, isoelectric focusing, amino acid analysis and amino acid sequencing. The molecular masses of UpII and III, as revealed by SDS-PAGE, were approximately 19 kDa in the presence of β-mercaptoethanol. However, in the absence of the reducing agent, both proteins migrated at much higher molecular masses (~40 kDa), more than twice the molecular weight in reducing conditions. This may be due to the presence of dimers linked by disulfide bonds. The calibration curve generated for estimation of their molecular weights is illustrated in Figure 6.4. The molecular masses of UpII and UpIII as shown by SDS-PAGE are illustrated in Figure 6.5.

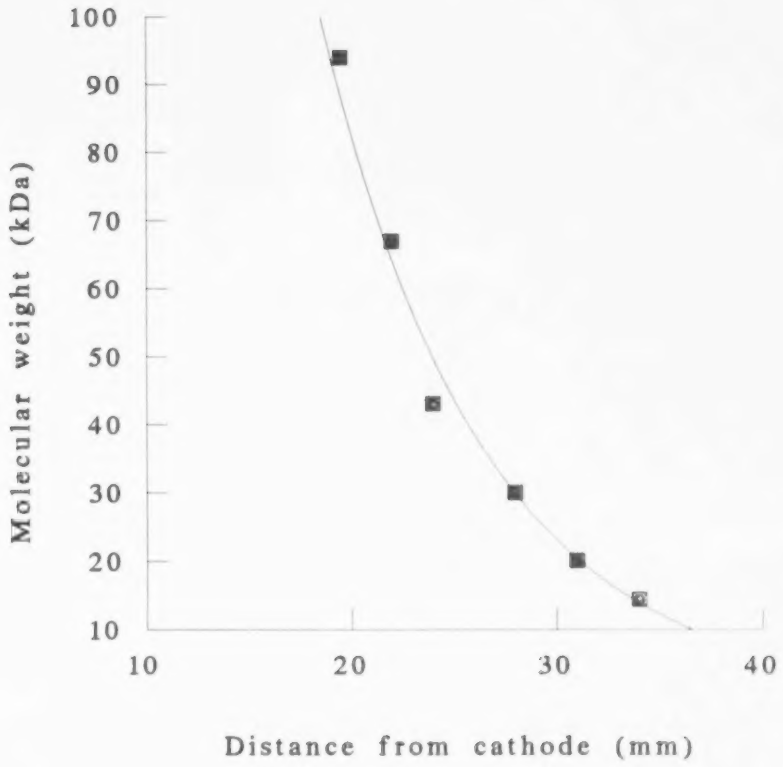
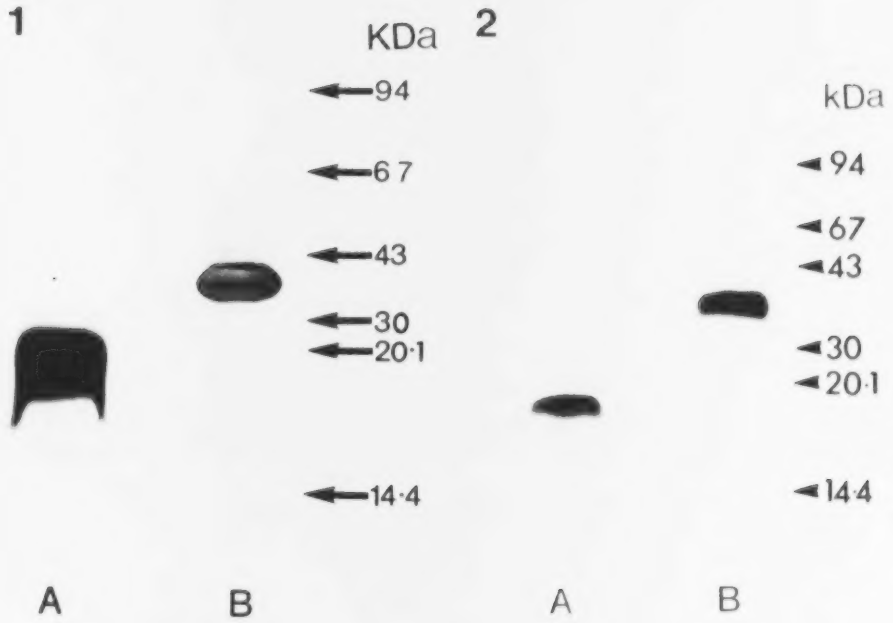


Figure 6.4 Calibration curve for molecular weight markers from SDS-PAGE (15%). Molecular markers are the same as in Fig. 5.5

Figure 6.5 Resolution of purified UpII (#1) and UpIII (#2) by SDS-PAGE, in the presence (A) and absence (B) of β-mercaptoethanol. The molecular weight markers are on the respective sides of the gel lanes.


1
KDa
2
94
67
43
30
20·1
14·4
kDa
94
67
43
30
20·1
14·4
A
B
A
B

Isoelectric focusing was carried out as described in Section 3.4.4.2. Based on the migration of the pI standards obtained from a calibration curve (Figure 6.6), the pI's of UpII and UpIII were estimated as 7.2 and 7.6, respectively. Figure 6.7 is an illustration of the isoelectric focusing gel. Their pI's indicate they are not basic, as is common for most cytolysins from sea anemones.

6.2.3.1 Amino acid composition of UpII and UpIII

Amino acid analyses revealed molecular weights of approximately 18.7 and 18.6 kDa respectively for both proteins (Table 6.1) and this correlates with the molecular weight obtained by SDS-PAGE. Both polypeptides had similar amino acid compositions except that UpII possessed one methionine residue while UpIII had none. While UpII was devoid of histidine and tyrosine, UpIII possessed 8 and 10 of these residues, respectively. Both proteins were rich in glycine, aspartic acid and glutamic acid residues. They were also rich in cysteine residues which accounted for 17.5 and 16.8 % of the total residues of UpII and UpIII, respectively.

SDS-PAGE was also used to locate the positions of cardiotonic activity within the gel and confirm that UpII and III were responsible for the activities observed. In a similar manner to UpI, cardiotonic activity was greatly reduced after electrophoresis. Cardiotonic activity profiles of both proteins after electrophoresis is illustrated in Figure 6.8.

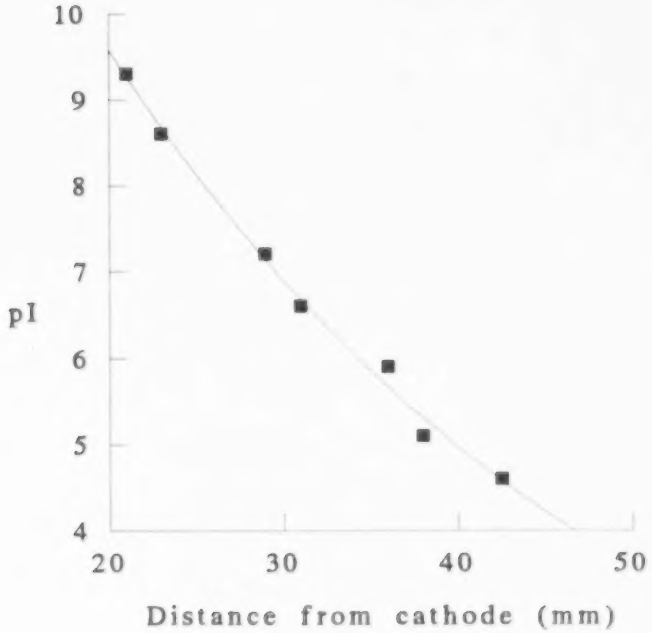
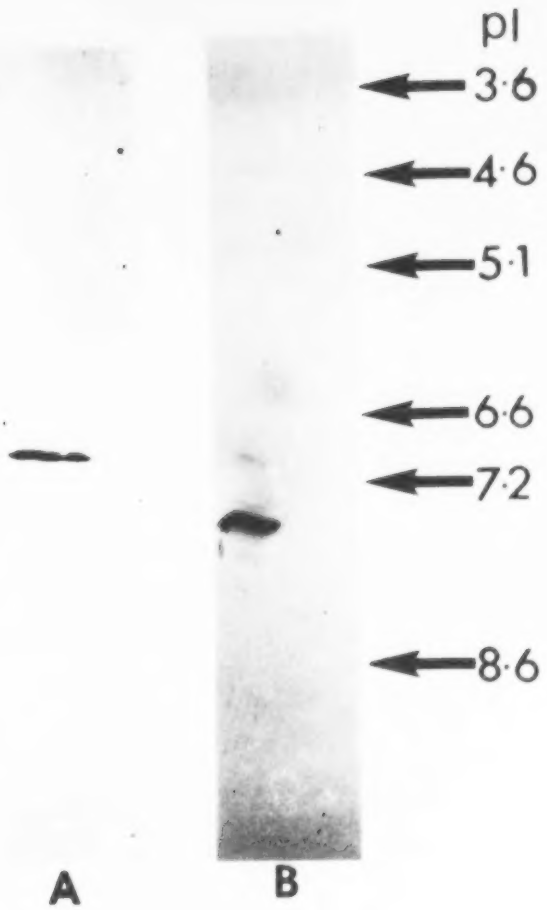


Figure 6.6 Calibration curve for pI markers from electrofocusing. pI markers are the same as in Fig. 5.7

Figure 6.7 Resolution of purified UpIII (A) and UpII (B) by analytical isoelectric focusing


pI
3·6
4·6
5·1
6·6
7·2
8·6
A
B

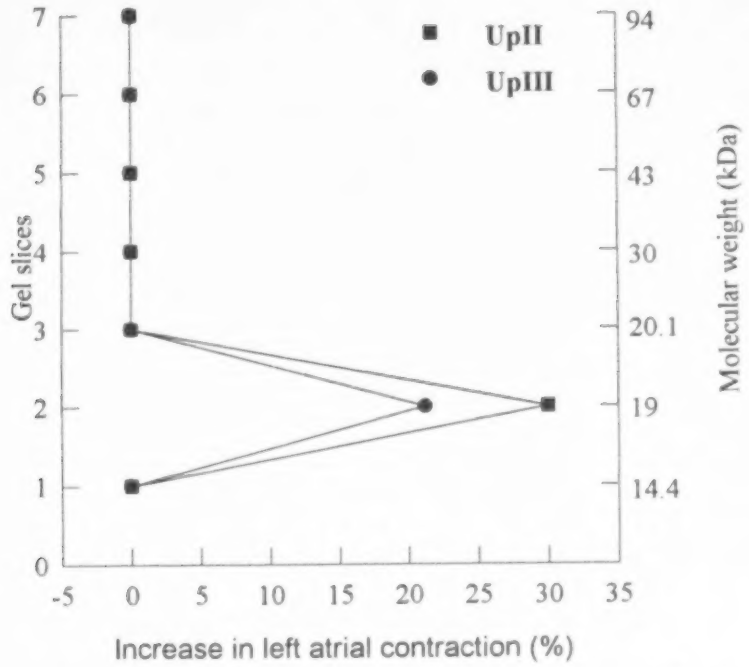


Figure 6.8 Plot of SDS-PAGE of reverse-phase purified UpII and UpIII against positive inotropic activity. Gels were prepared as described in Fig. 5.10. Activity was found only in the bands corresponding to UpII and UpIII.

Table 6.1 Amino acid composition of UpII and Up III

Amino acid	UpII Mole %	UpII No. of residues	UpIII Mole %	UpIII No. of residues
Asx	16.4	31.0 (31)	16.8	30.1 (30)
Thr	4.7	8.6 (9)	1.69	3.3 (3)
Ser	4.3	7.9 (8)	5.6	10.4 (10)
Glx	7.5	13.6 (14)	7.8	13.4 (14)
Pro	3.2	5.8 (6)	1.1	2.3 (2)
Gly	17.5	32.5 (33)	16.8	30.1 (30)
Ala	7.5	14.0 (14)	5.6	10.4 (10)
Cys	18.6	34.6 (35)	19.1	33.5 (34)
Val	4.7	8.7 (9)	4.5	8.4 (8)
Met	0.5	0.7 (1)	0	0
Ile	2.1	3.5 (4)	1.1	2.2 (2)
Leu	3.2	6.1 (6)	3.9	2.7 (3)
Tyr	0	0	4.5	8.1 (8)
Phe	0	0	0	0
His	0	0	5.6	10.4 (10)
Lys	6.4	12.3 (12)	3.9	7.4 (7)
Arg	3.2	5.6 (6)	3.9	6.9 (7)
Trp	nd	nd	nd	nd
Total residues		188		178
Mr		18,705		18,606

Values in parentheses represent the number of residues to the nearest integer, nd (not determined). Total number of amino acids and molecular weight calculated from the numbers in parentheses. Asx indicates asparagine or aspartic acid and Glx indicates glutamine or glutamic acid.

6.2.3.2 N-terminal sequencing of UpII and UpIII

The amino acid sequencing of both intact proteins was carried out until the 25 th cycle after which subsequent PTH-amino acids could not be discerned. The partial N-terminal sequences of both proteins are presented in Table 6.2. A search on Swiss-Prot[14] did not reveal any similar polypeptides or proteins from other sea anemones or elsewhere.

Table 6.2 N-terminal amino acid sequences of UpII and UpIII from *U. piscivora*

UpII	AT	D	K	W	NDCGS	VTAL	C	E	QK -	G	F	NKA	T	C
UpIII	D	D	D	W	DEGCH	VTAL	L	E	GQQ	G	R	NKA	A	C

Boxed letters indicate identical residues, (-) indicate gaps introduced by the program.

6.2.3 Evaluation of cardiac stimulatory and hemolytic activity

UpII and UpIII were purified by bioassay-guided separation using rat left atrial tissues. Both purified proteins elicited dose-dependent positive inotropic activity with EC_{50}'s of $1x10^{-7}$ and $7x10^{-6}$ M respectively. Tracings of the positive inotropic effects of UpII and III (10^{-6} M) are illustrated in Figure 6.9. UpII was, however, more potent and efficacious than UpIII although it was the minor protein component. The dose response relationship of UpII and III in relation to the positive inotrope INA is illustrated in Figure 6.10.

In an attempt to further characterize and ultimately classify UpII and III, they were both assayed for hemolytic activity using erythrocytes of rat, guinea pig and dog as described in Section 3.3.4. Both proteins were devoid of hemolytic activity, making them the first bioactive sea anemone proteins of molecular mass between 10-80 kDa that are devoid of hemolytic activity.

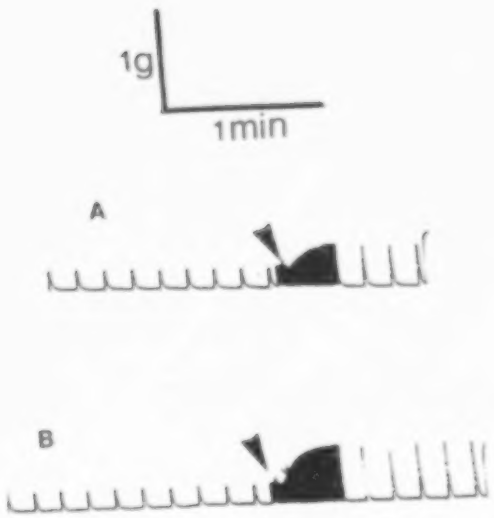


Figure 6.9 Tracing of the effects of UpII (A) and UpIII (B) ($1x10^{-7}$ M) on rat left atria. Resting tension 1 g. Arrow indicates point of addition of proteins

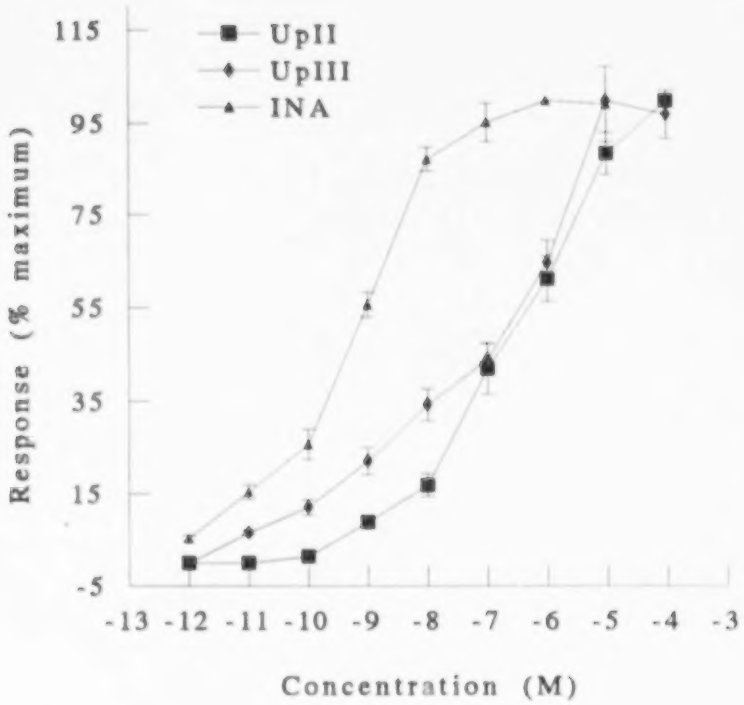


Figure 6.10 Dose response curves for UpII, UpIII and isoproterenol (INA). Values are means ± S.E.M. (n=3).

6.2.4 Discussion and conclusions

Described in this chapter is the purification and characterization of two new protein variants (isotoxins) from the sea anemone *Urticina piscivora*. The key to a successful separation was the use of a combination of chromatographic methods, particularly preparative reverse phase HPLC. It was this latter method that led to the resolution of such closely related proteins. Isotoxins from *Stichodactyla helianthus*,[4] *Actinia cari*,[10] *Actinia equina*[11] were all separated and purified using similar separation methods. However, isotoxins of *Heteractis magnifica* could only be separated using hydrophobic interaction chromatography.[13]

The nomenclature adopted for both proteins was similar to that of UpI, where the first letters of the genus and species were used with UpII being the second and UpIII the third protein purified. Unlike some known isotoxins with different molecular masses,[4,11] both proteins had similar molecular weights.

Based on the classification of sea anemone toxins,[2,3] UpII and III, by virtue of their high molecular weights, should be classified as cytolysins and their close biochemical similarity further classifies them as variants of eachother. However, all reported cytolysins to date possess hemolytic activity against erythrocytes of at least one mammalian species. Presented here are two proteins which lack hemolytic activity. This result clearly indicates that UpII and III do not belong to the already existing classes of sea anemone toxins. They appear to be a new class of cardiotonic polypeptides. Such results, and maybe others in

the future, will provide a basis for re-classification of the sea anemone polypeptides and proteins.

Many residues of UpII and III were identical to each other, and within the first 25 residues, 12 positions contained identical residues. If sequence homology is defined in terms of percentage of identical residues, then these two proteins show approximately 50 % homology over the regions for which sequence data are available. A better comparison can only be made when their respective primary structures are determined. The molecular weight of UpII and UpIII as shown by SDS-PAGE was 19 kDa in the presence of β-mercaptoethanol. In the absence of the reducing agent, they ran at molecular weights of 40 kDa, more than twice the original mass. This may be an indication that both proteins are dimeric a fact that is further strengthened by their high cysteine content as indicated in Table 6.1.

Although they were not hemolytic, which makes them good candidates for potential therapeutic evaluation, both proteins were less active than the potent but hemolytic UpI. A comparison of the properties of UpII and UpIII with other known protein variants is illustrated Table 6.3. Also, comparison of the N-terminal sequences of UpII and III and other known protein variants revealed no similarities. Sequences of cytolysin variants from sea anemones have been reported to be highly conserved, with similar amino acid residues in specific positions.[1,3,15] Although the physical properties of UpII and UpIII were not investigated, sea anemone isotoxins have been reported to show variable properties. For example, epiactin B from *Epiactis prolifera* is inactive at 50 °C whereas epiactin A is stable at this temperature.[8]

In conclusion, two closely related cardiac stimulatory protein variants have been isolated from *U. piscivora*. They are different biochemically and biologically from those that are already known. Based on these differences, UpII and UpIII may or may not have the same biological function with the organism.

Table 6.3 Comparison of the properties of UpII and UpIII to some other known protein variants

Sea anemone	Isotoxin	pI	Mol. wt	Cardiotonic Activity	Hemolytic Activity
Urticina piscivora	UpII	7.6	19,000	+	-
	UpIII	7.2	19,000	+	-
Stichodactyla helianthus[*]	ShI	8.7	17,600	-	+
	ShII	9.5	17,600	-	+
	ShIII	9.7	17,500	-	+
	ShIV	9.8	19,200	-	+
Actinia tenebrosa[⊥]	TN-A	9.8	19,800	+	+
	TN-B	Un	19,400	-	+
	TN-C	>10	20,200	+	+
Actinia cari[ϕ]	CTX I	9.4	19,800	-	+
	CTX II	10	19,800	-	+
Heteractis[#] *magnifica*	magnificalysin				
	I	9.4	19,000	-	+
	II	10	19,000	-	+

Un means unknown; (+) presence of stated activity; (-) absence of stated activity. References are * (Kem and Dunn[4]), ⊥ (Norton *et al.*[11]), ϕ (Sencic and Macek,[10]) and # (Khoo *et al.*[13]).

Bibliography

1. Kem, W. R. (1988) Sea anemone toxins: structure and action. In: *Biology of Nematocysts.* (Hessinger, D. A. and Lenhoff, H. M. eds.) pp. 375-405, Academic Press Inc. New York.

2. Macek, P. (1992) Polypeptide cytolytic toxins from sea anemones (Actinaria). *FEMS Microbiol. Immunol.* **105**, 121-130.

3. Turk, T. (1991) Cytolytic toxins from sea anemones. *J. Toxicol.-Toxin Rev* **10**, 223-262.

4. Kem, W. R. and Dunn, B. M. (1988) Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon* **26**, 997-1008.

5. Macek, P. and Lebez, D. (1988) Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L. *Toxicon* **26**, 441-451.

6. Ferlan, I. and Lebez, D. (1974) Equinatoxin, a lethal protein from *Actinia equina*-I. Purification and characterization. *Toxicon* **12**, 57-61.

7. Komatsu, K., Furukawa, K., Abe, K., Hirano, H. and Ueda, M. (1992) Isolation and characterization of equinatoxins from the sea anemone *Actinia equina* L. *Chem. Pharm. Bull.* **40**, 2873-2875.

8. Bernheimer, A. W. and Avigad, L. S. (1982) Toxins of sea anemone *Epiactis prolifera*. *Arch. Biochem. Biophys.* **217**, 174-180.

9. Macek, P., Sencic, L. and Lebez, D. (1982) Isolation and partial characterization of three lethal and hemolytic toxins from the sea anemone *Actinia cari*. *Toxicon* **20**, 181-185.

10. Sencic, L. and Macek, P. (1990) New method for isolation of venom from the sea anemone *Actinia cari*. Purification and characterization of cytolytic toxins. *Comp. Biochem. Physiol.* **97B**, 687-693.

11. Norton, R. S., Bobek, G., Ivanov, B. J., Thomson, M., Fiala-Beer, E., Moritz, R. L. and Simpson, R. J. (1990) Purification and characterization of proteins with cardiac stimulatory and haemolytic activity from the sea anemone *Actinia tenebrosa*. *Toxicon* **28**, 29-41.

12. Simpson, R. J., Reid, G. E., Moritz, R. L., Morton, C. and Norton, R. S. (1990) Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and hemolytic protein from the sea anemone *Actinia tenebrosa*. *Eur. J. Biochem.* **190**, 319-328.

13. Khoo, K. S., Kam, W. K., Khoo, H. E., Gopalakrishnakone, P. and Chung, M. C. M. (1993) Purification and partial characterization of two cytolysins from a tropical sea anemone *Heteractis magnifica*. *Toxicon* **31**, 1567-1579.

14. Bernheimer, A. W., Avigad, L. S., Branch, G., Dowdle, E., and Lai, C. Y. (1984) Purification and properties of a toxin from the South African sea anemone, *Pseudactinia vari*. *Toxicon* **22**, 183-191.

15. Wunderer, G., Machleidt, W., and Watcher, E.,(1976) Toxin II from *Anemonia sulcata* - the first sequence of a coelenterate toxin. *Hoppe-Sey. Z. Physiol. Chem.* **357**, 239-240.

VII. BIOLOGICAL ACTIVITY OF PROTEINS UPI, UPII AND UPIII FROM *U. PISCIVORA*[*]

7.1 Introduction

Many researchers have established the fact that sea anemones possess polypeptides and protein toxins with different biological activities.[1-3] Five major activities have been identified to date. They are hemolytic,[1-3] neurotoxic,[4-6] cytotoxic,[7,8] antitumor[9] and cardiac stimulatory activities.[10-12]

In 1988, Kem[1] proposed the name "actinoporins" for the group of polypeptides with mass 10-30 kDa. These toxins have been reported to possess pore forming activities in natural and lipid membranes.[13,14]

The phospholipid sphingomyelin has since been reported as the receptor molecule for these toxins.[15] The only exception has been metridiolysin from the sea anemone *Metridium senile*, whose hemolytic activity is inhibited by cholesterol.[16] Although there is no definitive understanding of the mechanism of action of these toxins, it is believed that pore formation is induced by colloid osmotic cell damage.[15,17]

Besides their primary hemolytic activity, some sea anemone cytolysins are also cytotoxic.[3] However, only a few reports describing their cytotoxic activity exist so far.[3,7,9] Morphological evidence to support the cytotoxic activity of equinatoxin II (EqT II), from *Actinia equina*, was revealed by transmission electron microscopy, which showed ultrastructural changes to plasma membrane and cell morphology.[18]

---

[*] A version of this chapter has been accepted for publication. Cline *et al.* **Pharmacological Res**. (In Press).

Screening of crude extracts of sea anemones as highlighted in chapter 4, revealed that the extracts possessed potent hemolytic activity to erythrocytes of three mammalian species. Extracts of *Urticina piscivora* were some of the most potent hemolytic and cytotoxic extracts (Table Figures 4.5-4.7 and Table 4.4). In an attempt to determine if the proteins purified from *U. piscivora* could be responsible singly or synergistically for some of the activities observed with the crude extracts, their hemolytic, cytotoxic and antifungal effects were evaluated.

7.2 Results

7.2.1 Hemolytic activity of proteins

The *in vitro* hemolytic activity of UpI, II and III was evaluated as described (Section 3.3.4) using erythrocytes of five mammalian species (rat, guinea pig, dog, pig and human). UpII and UpIII were devoid of hemolytic activity. UpI on the other hand, elicited potent hemolytic action against all erythrocytes, with sensitivity increasing in the order pig > rat > dog > guinea pig > human. A sigmoidal dose-dependent hemolysis was observed with the minimum concentration at which hemolysis was observed being 10^{-10} M. The hemolytic action of UpI on these erythrocytes is illustrated in Figure 7.1. The ability of sphingomyelin (2.5 and 25 μg/ml) to inhibit UpI-induced (10^{-5} M) hemolysis was investigated. Hemolytic activity was inhibited in a dose-dependent manner by sphingomyelin, by as much as 20 % and 75 % of the control activity. The extent of inhibition of UpI induced hemolysis by sphingomyelin is illustrated in Figure 7.2.

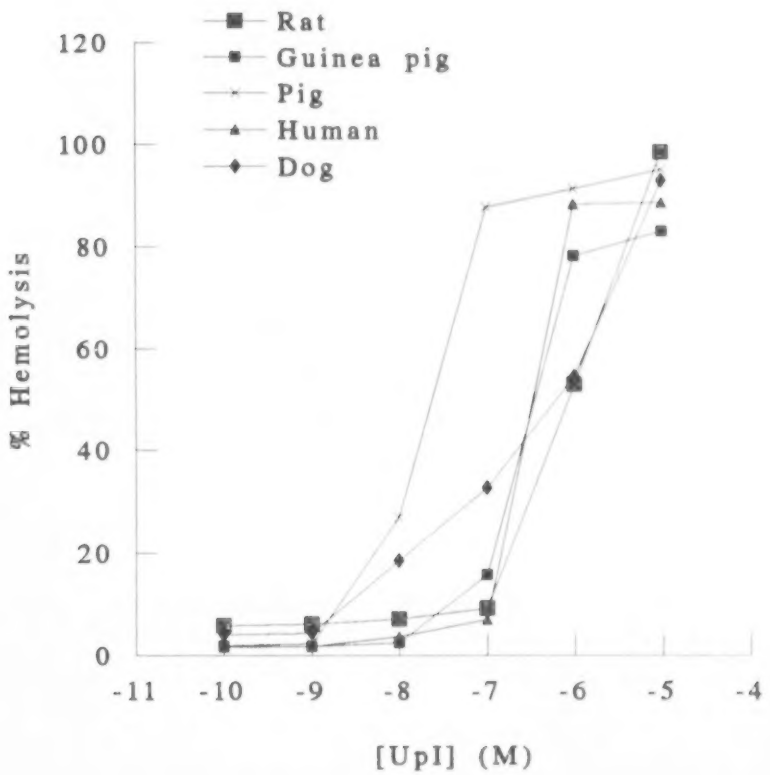


Figure 7.1 Relative cytolytic activities of UpI measured on erythrocytes of five mammalian species.

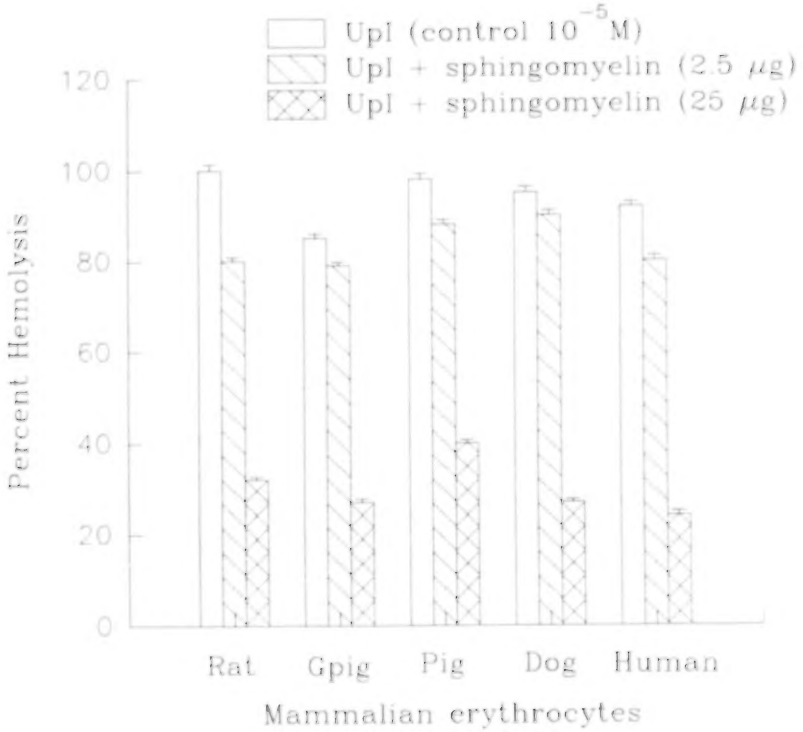


Figure 7.2 Effect of different concentrations of the phospholipid sphingomyelin on hemolytic action of UpI using different mammalian erythrocytes. Values are mean ± S.E.M. (n=3). Gpig (guinea-pig).

7.2.2 Scanning electron microscope (SEM) studies

The use of SEM to investigate morphological damage, if any, to the membranes of rat and guinea pig erythrocytes is described Section 3.4.5. UpI caused substantial structural damage to the membranes, which in turn may have caused the hemolysis observed within 5 min. of addition of the toxin to the suspensions. Numerous holes and breaks were observed on both membranes but not the control membranes as indicated in Figure 7.3. UpII and UpIII which were not hemolytic, were not investigated

7.2.3 Cytotoxic activity of proteins

Only UpI was investigated for cytotoxic activity. Although toxic to the three cell lines used in the assay, it was not as potent as the crude extract of *U. piscivora* (Table 7.1). The high potency of the crude extract may be due to the effect of other polypeptides and proteins contained within the extracts. The results also confirm that UpI may be one of the proteins responsible for the cytotoxic action observed for the crude extract. The crude extract was in the range of 2-10 times more potent than the pure protein on individual cell lines. A comparison of the cytotoxic action of the crude extract and UpI is illustrated in Table 7.1.

Figure 7.3 Scanning electron microscopic appearance of membranes derived from distilled water (control) (A and C) and UpI treated rat (B) and guinea pig (D) erythrocytes. Membranes were prepared as described (Section 3.4.5). Note the numerous breaks and holes on both test membranes as indicated by arrows. For figures A and B the line represents 0.5 μm and for figures C and D, the line represents 1.0 μm.

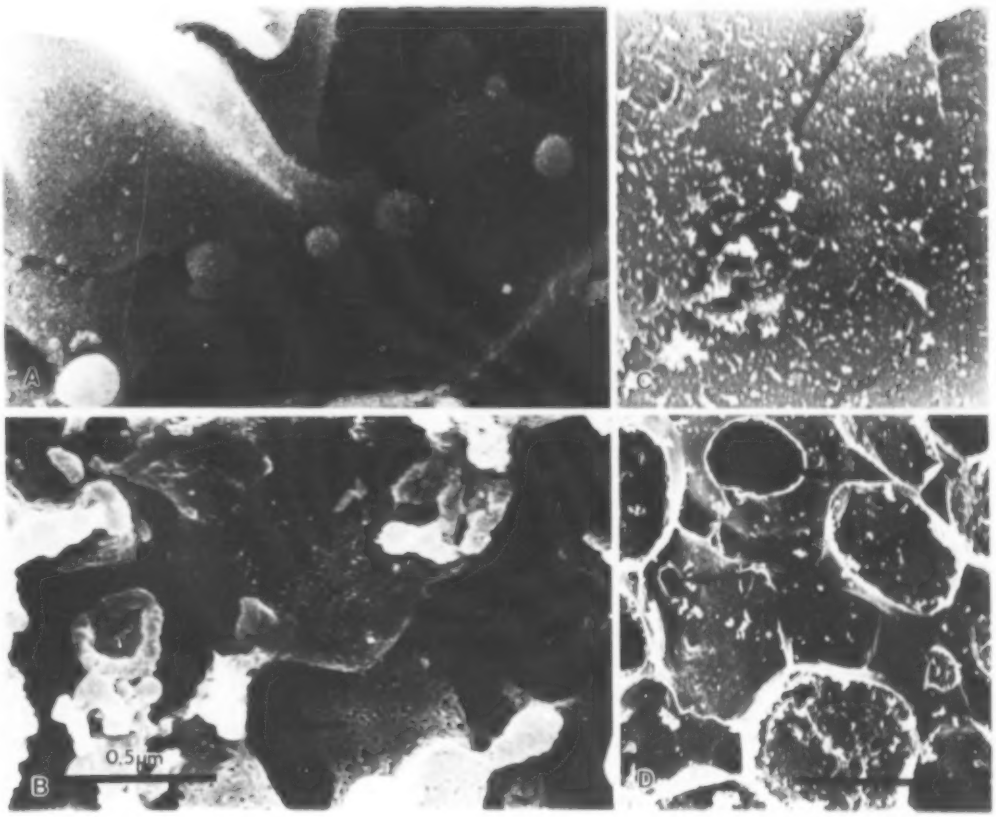

A
C
B
0.5 µm
D

Table 7.1 Cytotoxic effect of crude extract of *U. piscivora* and UpI

Test product	TC_{50} (µg/ml) KB cell	L1210	HEL 299
Crude extract (*U. piscivora*)	6.54	10.07	2.34
UpI	40.32	29.99	29.74

TC_{50} concentration (µg/ml) of test product that inhibited the growth of 50 % of cells.

UpII and UpIII were not assayed.

7.2.4 Antifungal activity of proteins

All three proteins were devoid of antifungal activity against test cultures of *Candida albicans* and *Aspergillus niger*. Absence of antifungal activity is consistent with results obtained with the crude extracts. The absence of these fungal species in the marine environment may account for the lack of any antifungal activity.

7.3 Discussion and conclusions

The present results clearly demonstrate that UpI possesses potent hemolytic and moderate cytotoxic activities. This is consistent with the activities observed for the crude extract of *U. piscivora* from which it was isolated. The other cardiotonic proteins were not evaluated.

The cytotoxic activity of only one sea anemone toxin, equinatoxin (EqT II), has been investigated both *in vitro* and *in vivo*. However, it is less potent *in vivo* when compared to its *in vitro* activity. It has been suggested that this may be due to poor systemic absorption and neutralizing activity exerted by serum phospholipids, proteases[9] and body fluids.[7] EqT II produces extensive lysis of tumor cells as observed by phase contrast microscopy.[9]

The hemolytic action of UpI on all five species of erythrocytes was sigmoidal in nature (Figure 7.1). This is consistent with hemolytic activities observed for other anemone cytolysins. Hemolysis by cytolysins is said to occur in three phases, viz.: an initial lag time or induction period, a linear period of rapid lysis and a termination period during which the remaining cells hemolyse.[20] The sensitivity order for the erythrocytes were pig > rat > dog > guinea pig > human which was consistent with the crude extract of *U. piscivora* where the sensitivity order was rat > dog > guinea pig, as only three erythrocytes were tested. Similar susceptibility orders have been reported for the cytolysin

Sh III from *Stoichactis helianthus* where the order of sensitivity was guinea pig > horse > human > rat > dog > sheep > rabbit > cat erythrocytes.[21]

The ability of UpI to lyse mammalian erythrocytes, plus the inhibition of hemolysis by sphingomyelin, is consistent with the suggestion that sea anemone cytolysins are channel-forming polypeptides affecting mainly the phospholipid part of biological membranes.[2,13,17,19] Sphingomyelin might be the sole receptor or one of the receptor molecules for UpI. Belmonte and his co-workers,[17] have since shown, using model lipid membranes and human red blood cells, that EqT II-induced pores on these cells with a radius of about 1.1 nm.

The only cytolysin known to behave differently is metridiolysin from *Metridium senile*, which acts similarly to the thiol-activated bacterial cytolysins and is inhibited by cholesterol.[13] In spite of the fact that sphingomyelin can inhibit the cytolytic action of sea anemones, the amount required to achieve total inhibition varies from one toxin to the next. Isotoxins have also been reported to show different susceptibility to sphingomyelin inhibition.[3] At the two concentrations tested, sphingomyelin did not completely inhibit hemolysis. The concentration required to bring about complete hemolysis has been reported to vary among the cytolysins. For example, 30 % hemolysis was observed for caritoxin I (CTX I) from *Actinia caria*, at a sphingomyelin concentration of 3 μg/ml.[5] Similar results were obtained from epiactin A and B.[22]

Although there is still no definitive understanding of the mechanism of action of sea anemone cytolysins,[17] distinct morphological damage has been known to follow treatment with metridiolysin.[23] Examination of the membranes of rat and guinea pig by

SEM revealed numerous holes and breaks in the membranes. While studies with variolysin from *Psuedactinia vari* did not reveal any structural damage to sheep erythrocytes[24] the same erythrocytes treated with metridiolysin revealed ring-like structures on its membranes.[23] The action of UpI on the membranes is similar to that produced by the bungarotoxins, known for their phospholipase activity.[25] Based on the potent hemolytic action of UpI, its sequence similarity to the bungarotoxins and a molecular weight similar to toxins from the sea anemone *Aiptasia pallida* (known for its PLA_2-like action), it is possible that UpI also exerts its toxic effect via phospholipase activity.

These results clearly indicate that UpI is hemolytic and brings about structural damage to membranes of mammalian erythrocytes. However the biochemical mechanism for hemolysis cannot be commented on at this time, since further studies are required for this to be done.

Bibliography

1. Kem, W. R. and Dunn, B. M. (1988) Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon* **26**, 997-1008.

2. Macek, P. (1992) Polypeptide cytolytic toxins from sea anemones (Actinaria). *FEMS Microbiol. Immunol.* **105**, 121-130.

3. Turk, T. (1991) Cytolytic toxins from sea anemones. *J. Toxicol.-Toxin Rev.* **10**, 223-262.

4. Beress, L., Beress, R. and Wunderer, G. (1975) Purification of three polypeptides with neuro- and cardiotoxic activity from the sea anemone *Anemonia sulcata*. *Toxicon* **13**, 359-367.

5. Cariello, L., de Santis, A., Fiore, F., Piccoli, R., Spagnuolo, S., Zanetti, L. and Parente, A. (1989) Calitoxin, a neurotoxic peptide from the sea anemone *Calliactis parasitica*: amino acid sequence and electrophysiological properties. *Biochem.* **28**, 2484-2489.

6. Kem, W. R., Parten, B., Pennington, M.W., Price, D. A. and Dunn, B. M. (1989) Isolation, characterization and amino acid sequence of a polypeptide neurotoxin occurring in the sea anemone *Stichodactyla helianthus*. *Biochem.* **28**, 3483-3489.

7. Batista, U., Macek, P. and Sedmak, B. (1990) The cytotoxic and cytolytic activity of equinatoxin II from the sea anemone *Actinia equina*. *Cell Biol. Int. Rep.* **14**, 1013-1024.

8. Zorec, R., Tester, M., Macek, P. and Mason, W. T. (1990) Cytotoxicity of equinatoxin II from the sea anemone *Actinia equina* involving channel formation and an increase in intracellular calcium activity. *J. Memb. Biol.* **118**, 243-249.

9. Giraldi, T., Ferlan, I. and Romeo, D. (1976) Antitumor activity of equinatoxin. *Chem.-Biol. Interactions.* **13**, 199-203.

10. Alsen, C., Peters, T. and Scheufler, E. (1982) Studies on the mechanism of the positive inotropic effect of ATX II (*Anemonia sulcata*) on isolated guinea pig atria. *J. Cardiovas. Pharmacol.* **4**, 63-69.

11. Galettis, P. and Norton, R. S. (1990) Biochemical and pharmacological studies of the mechanism of action of tenebrosin C, a cardiac stimulatory and hemolytic protein from the sea anemone, *Actinia tenebrosa*. *Toxicon* **28**, 695-706.

12. Shibata, S., Norton, T. R., Izumi, T. and Katsuki, S. (1976) A polypeptide (AP-A) from sea anemone (*Anthopleura xanthogrammica*) with potent positive inotropic action. *J. Pharmacol. Exp. Ther.* **199**, 298-309.

13. Bernheimer, A. W. and Rudy, B. (1986) Interactions between membranes and cytolytic peptides. *Biochim. Biophys. Acta* **864**, 123-141.

14. Harvey, H. L. (1990) Cytolytic toxins. In: *Handbook of Toxinology* (Shier, W. T. and Mebs, D. eds.) pp. 1-66, Marcel Dekker, New York.

15. Suput, D. (1994) Equinatoxin II and Exocytosis. *Toxins and Exocytosis*. *Ann. N. Y. Acad. Sc.* **710**, 30-37.

16. Bernheimer, A. W. and Avigad, L. (1978) A cholesterol-inhibitable cytolytic protein from the sea anemone *Metridium senile*. *Biochim. Biophys. Acta* **541**, 96-106.

17. Belmonte, G., Pederzolli, C., Macek, P. and Menestrina, G. (1993) Pore formation by sea anemone cytolysin equinatoxin II in red blood cells and model lipid membranes. *J. Memb. Biol.* **131**, 11-22.

18. Batista, U., Jezernik, K., Macek, P. and Sedmak, B. (1987) Morphological evidence of cytotoxic and cytolytic activity of equinatoxin II. *Period. Biolog.* **89**, 347-348.

19. Michaels, D. W. (1979) Membrane damage by a toxin from the sea anemone *Stoichactis helianthus*. 1. Formation of transmembrane channels in lipid bilayers. *Biochim. Biophys. Acta* **555**, 67-78.

20. Hessinger, D. A., Lenhoff, H. M. and Kahan, L. B. (1973) Hemolytic, phospholipase A and nerve-affecting activities of sea anemone nematocyst venom. *Nature New Biol.* **241**, 125-127.

21. Macek, P., Belmonte, G., Pederzolli, C. and Menestrina, G. (1994) Mechanism of action of equinatoxin II, a cytolysin from the sea anemone *Actinia equina* L. belonging to the family of actinoporins. *Toxicol.* **87**, 205-227.

22. Bernheimer, A. W. and Avigad, L. S. (1982) Toxins of sea anemone *Epiactis prolifera*. *Arch. Biochem. Biophys.* **217**, 174-180.

23. Bernheimer, A. W., Avigad, L. S. and Kim, K. (1979) Comparison of metridiolysin from the sea anemone with thiol-activated cytolysins from bacteria. *Toxicon* **17**, 69-75.

24. Bernheimer, A. W., Avigad, L. S., Branch, G., Dowdle, E. and Lai, C. Y. (1984) Purification and properties of a toxin from the South African sea anemone, *Pseudactinia vari*. *Toxicon* **22**, 183-191.

VIII. PATHOPHYSIOLOGICAL ACTION OF SEA ANEMONE EXTRACTS AND PROTEINS FROM *URTICINA PISCIVORA*[*]

8.1 Introduction

Sea anemones are colorful Cnidarians of varying sizes, occurring in all oceans from the inter-tidal zone to depths of about 900 m. As stated previously, they discharge their toxic components into their prey via the nematocysts.[1]

Contact dermatitis from sea anemones is one of the most frequent causes of reactions to coelenterates and is seen in sea snails or crab searchers and bathers during the summer.[2] Sea anemone stings tend to be localized in their effects, with initial symptoms ranging from prickly sensations over the affected area to burning pain. The latter occurs immediately on contact and increases over the next few minutes before gradually diminishing over hours or days.[3] Additional symptoms may also appear the same day, or several days after exposure, and include lesions, itching with subsequent necrosis and severe ulcerations.[3,4]

A variety of sea anemones have been reported as inflicting painful stings.[5] They include *Actinodendron plumosum* (hell's fire sea anemone), which produces severe ulcerations.[3] The anemone *Anemonia sulcata* was reported to have caused erythema, pruritus and burning sensations to the arms and knees of a fifty-three year old fisherman

---

[*] A version of this chapter is in preparation for publication.

along a sea shore in Spain. Seven other cases of contact dermatitis due to this species and *A. equina* have been reported.[2,6]

Although the biochemical nature of the polypeptide toxins of some sea anemones has been investigated,[7,8] very few have been evaluated for their pathophysiological actions in mammalian species. Only equinatoxin II (EqT II) from *Actinia equina* have been investigated for this particular action.[9-11] EqT II has been shown to cause cardiac arrest, coronary vasospasm and other cardiorespiratory effects.[12]

In these studies the toxic effects of crude extracts of seven species of sea anemones, (*Anthopleura xanthogrammica, Pachycerianthus fimbratus, Stomphia didemon, Urticina coriacea, U. crassicornis, U. lofotensis and U. piscivora*) together with the three proteins (UpI, UpII and UpIII), isolated from *U. piscivora*, were determined in anesthetized rats. The toxic effects elicited by UpI were shown to be consistent with those of the crude extract from which it was obtained.

The pathophysiological changes induced by these compounds and the morphological and structural changes induced by them are reported in this chapter. UpII and UpIII and with the crude extract of *Pachycerianthus fimbratus* were inactive.

8.2 Results

8.2.1 Isolated smooth muscle experiments

All extracts tested and UpI induced contractile responses of isolated, longitudinal smooth muscle strips. UpII and III were inactive. The concentration that induced

maximal contraction was 25 mg/ml for the extracts and 10^{-5} M for UpI. Higher concentrations either caused no further contraction or decreased its size.

All contractile responses were reduced or abolished by atropine. Figure 8.1 shows the maximum contractile responses achieved for each extract and the decrease induced by atropine. Tetrodotoxin did not alter any contractile responses, showing that the extracts and UpI do not act via cholinergic nerves but solely by muscarinic receptors. The dose-response relationships of ACh and UpI and the inhibition by atropine are shown in Figures 8.2 and 8.3, respectively. Tracings showing the contractile responses are also shown (Fig. 8.4). Whereas ACh produced a biphasic contractile response, the response elicited by UpI was monophasic.

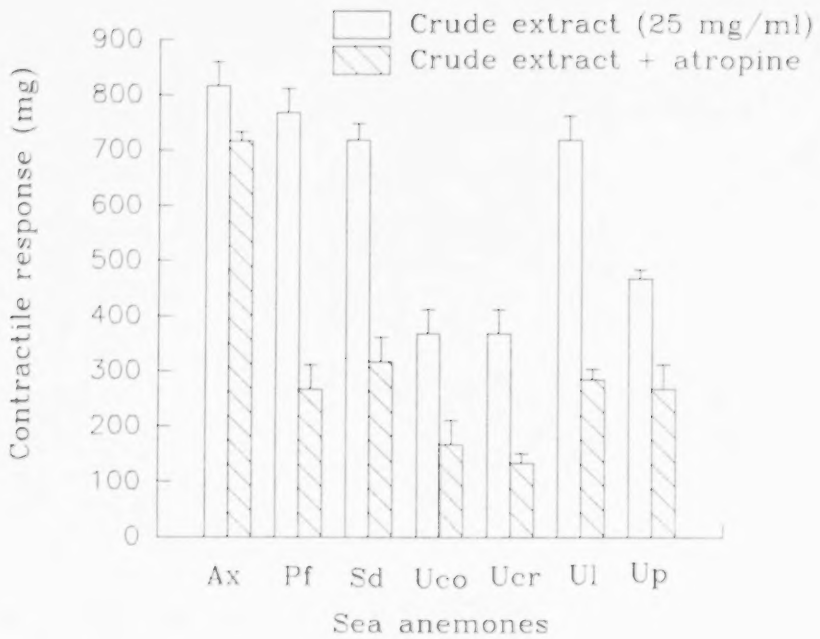


Figure 8.1 Contractile effects of crude extract of sea anemones on guinea-pig longitudinal smooth muscle. **Ax (*A. xanthogrammica*), Pf (*P. fimbratus*), Sd (*S. didemon*), Uco (*U. coriacea*), Ucr (*U. crassicornis*), Ul (*U. lofotensis*), Up (*U. piscivora*). Values are mean ± S. E. M. (n=3).**

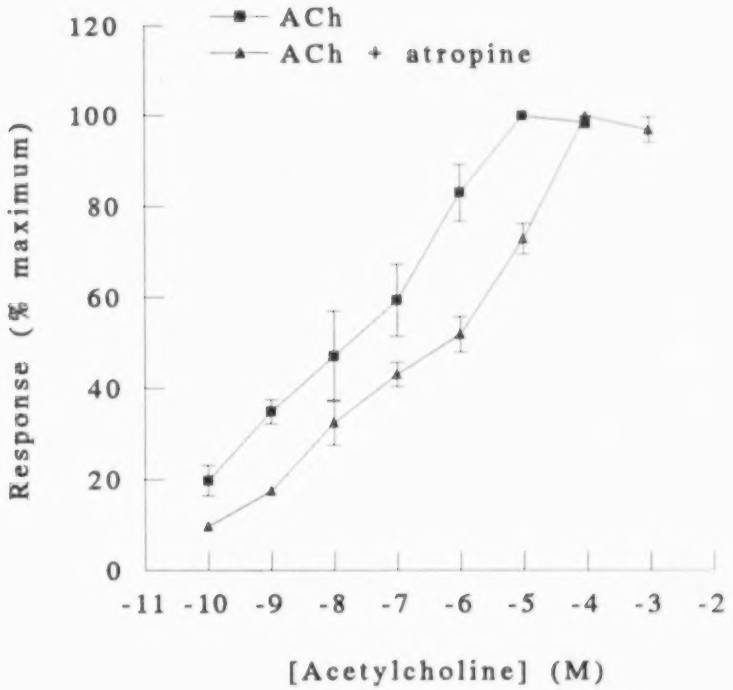


Figure 8.2 Effect of acetyl choline alone and with atropine on isolated guinea-pig ileum. Values are mean ± S.E.M (n=3) (ACh = acetylcholine).

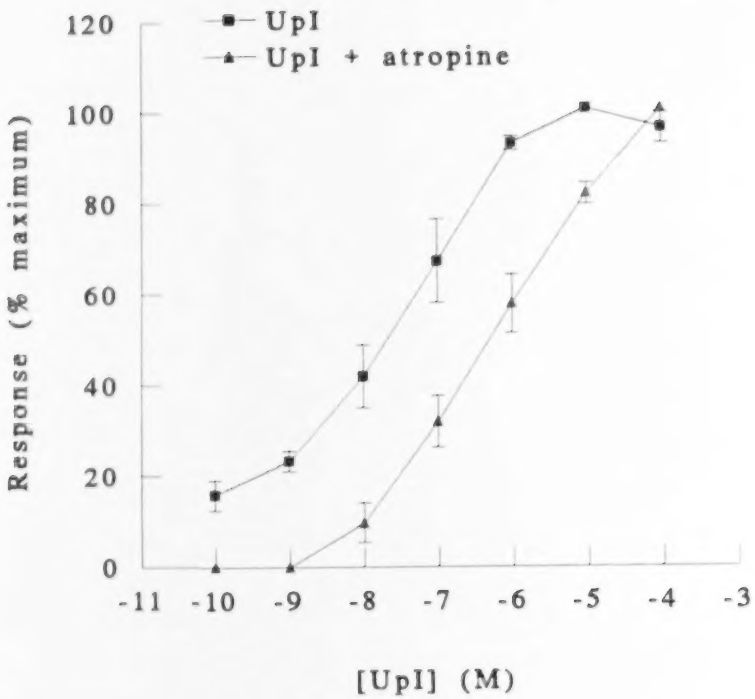


Figure 8.3 Effect of UpI alone and with atropine on guinea-pig ileum. Values are mean ± S.E.M. (n=3).

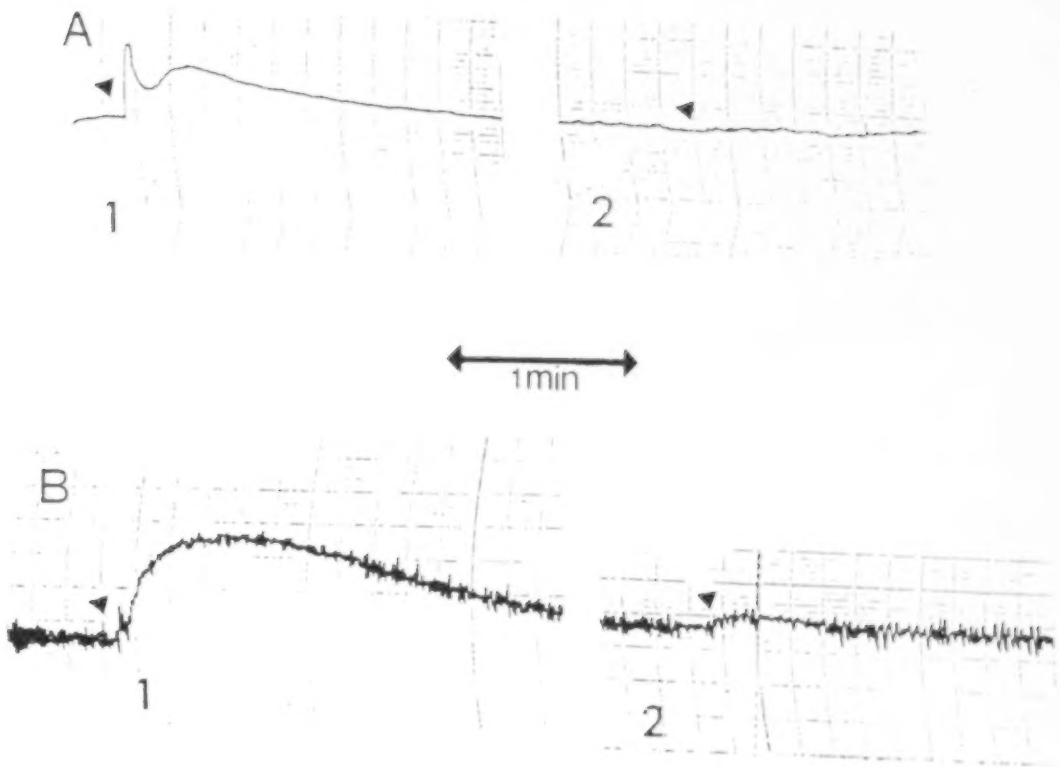


Figure 8.4 Tracings of the contractile action of acetyl choline (A) 10^{-5} M and UpI (B) 10^{-5} M on guinea-pig longitudinal smooth muscle. A2 & B2 shows the effects of the agonists in the presence of atropine (10^{-5} M). Note inhibition of contraction. Arrow shows point of addition of test substances.

8.2.2 Effects on blood pressure (BP) and heart rate (HR)

All extracts and UpI produced a fall in arterial blood pressure (Figures 8.5A - 8.11A). Intravenous injection of the extracts and UpI induced respiratory dysrhythmia which manifested themselves in the form of gasping, labored breathing and extrusion of fluid from the nostrils and the cannulated trachea.

In atropinized animals, the fall in arterial blood pressure was reduced but not abolished (Figures 8.5 - 8.11). Rats died at much higher doses, at which time bradycardia was observed. Hypotension and bradycardia may have been provoked by an increased vagal discharge. After pretreatment of rats with atropine, unlike the effect observed for all extracts, the effect of extracts of *S. didemon* were abolished by atropine and a gradual rise in arterial pressure was observed (Figure 8.7). In this case, rats survived even at concentrations as high as 100 µg/kg. All extracts induced an initial increase in heart rate which remained constant until bradycardia set in (Figures 8.5 - 8.11B)

The effect of atropine on cardiovascular responses to UpI was reduced also evaluated. Hypotension was reduced but abolished. The initial increase in heart rate was much higher and remained constant until bradycardia and subsequent respiratory arrest (Figure 8.11).

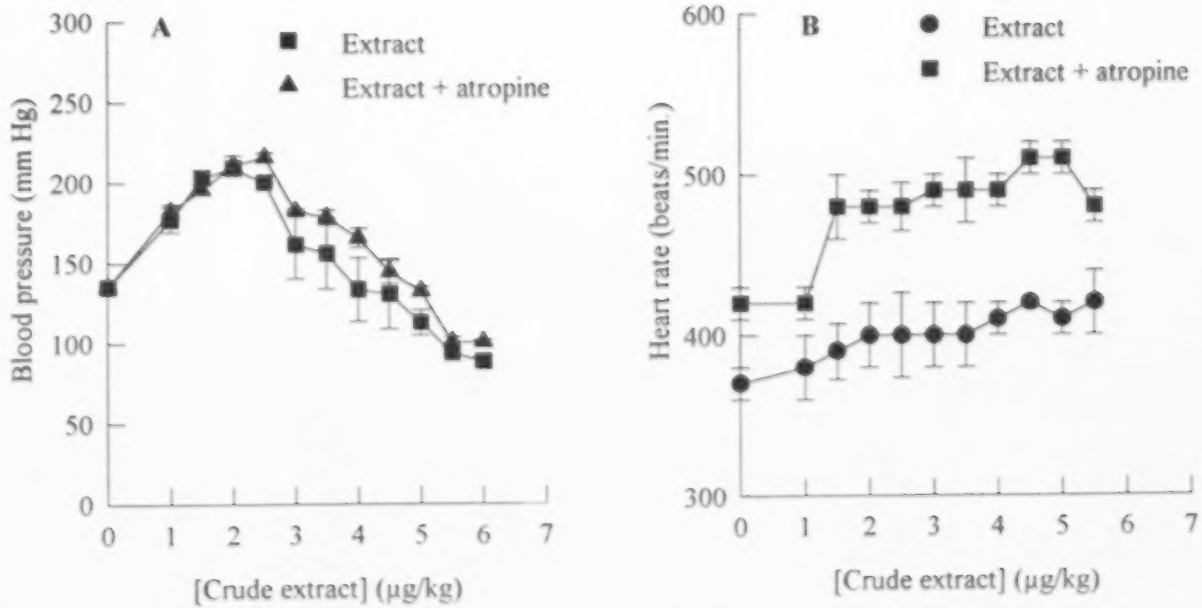


Figure 8.5 Effect of crude extract of *A. xanthogrammica* on arterial blood pressure (A) and heart rate (B) of anesthetized rats. Values are mean ± S.E.M. (n=3).

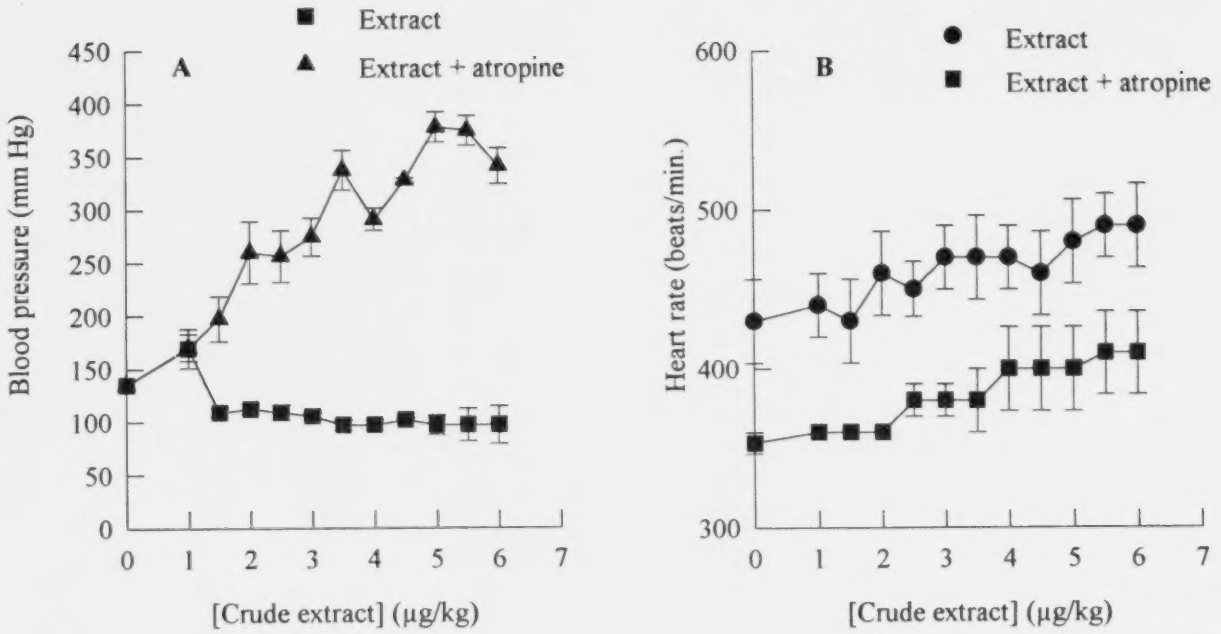


Figure 8.6 Effect of crude extract of *S. didemon* on arterial blood pressure (A) and heart rate (B) of anesthetized rats. Values are mean ± S.E.M. (n=3).

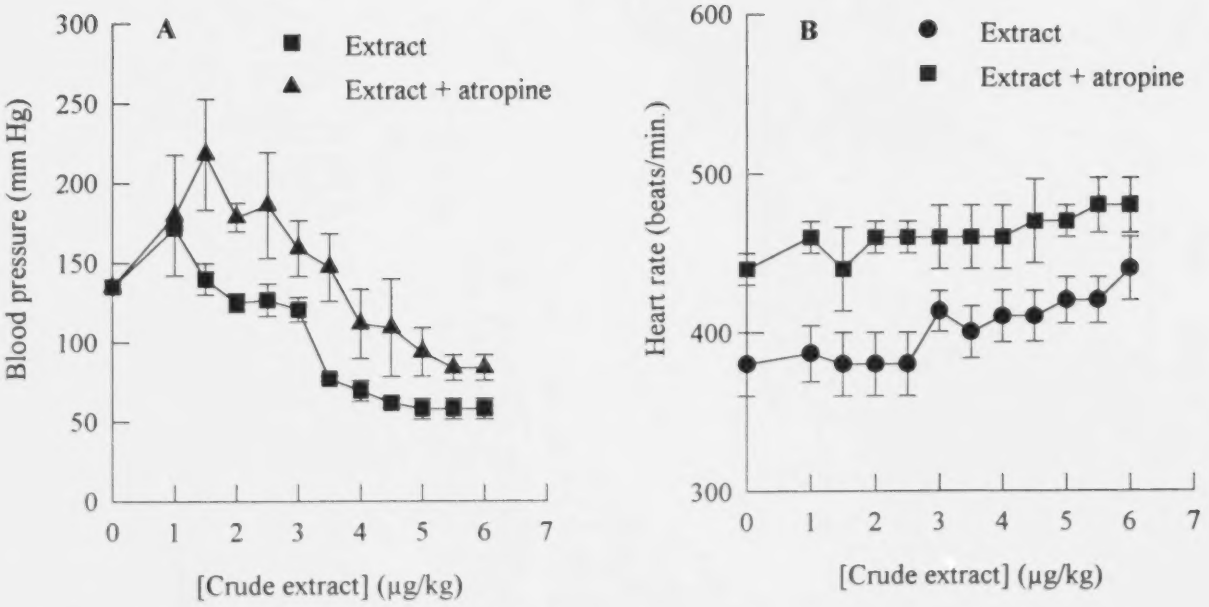


Figure 8.7 Effect of crude extract of *U. coriacea* on arterial blood pressure (A) and heart rate (B) of anesthetized rats. Values are mean ± S.E.M. (n=3).

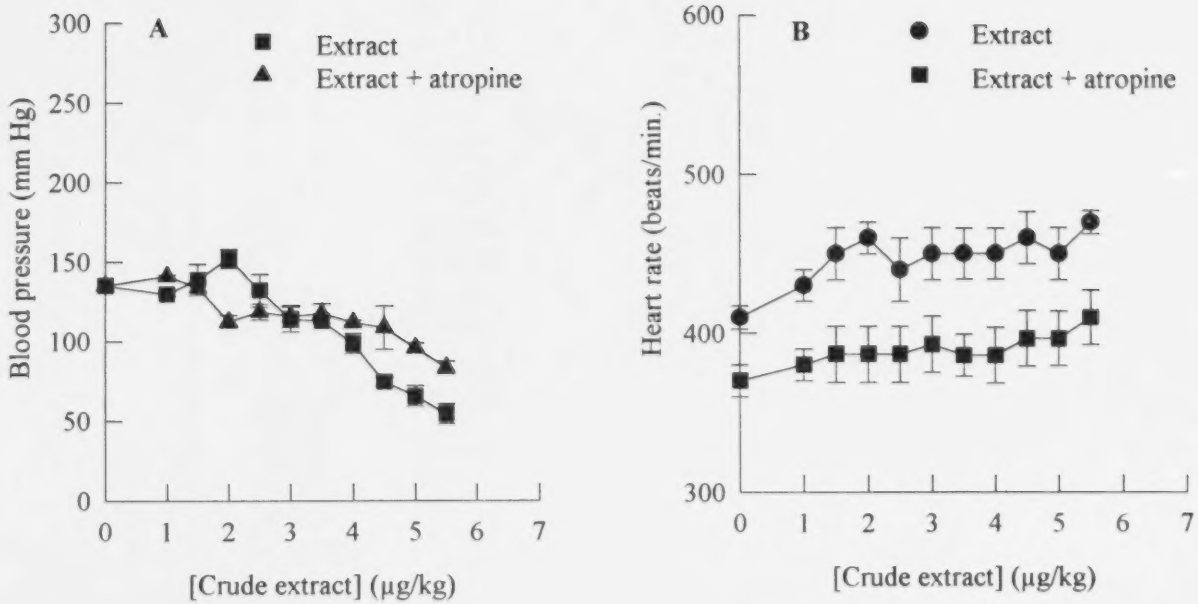


Figure 8.8 Effect of crude extract of *U. crassicornis* on arterial blood pressure (A) and heart rate (B) of anesthetized rats. Values are mean ± S.E.M. (n=3).

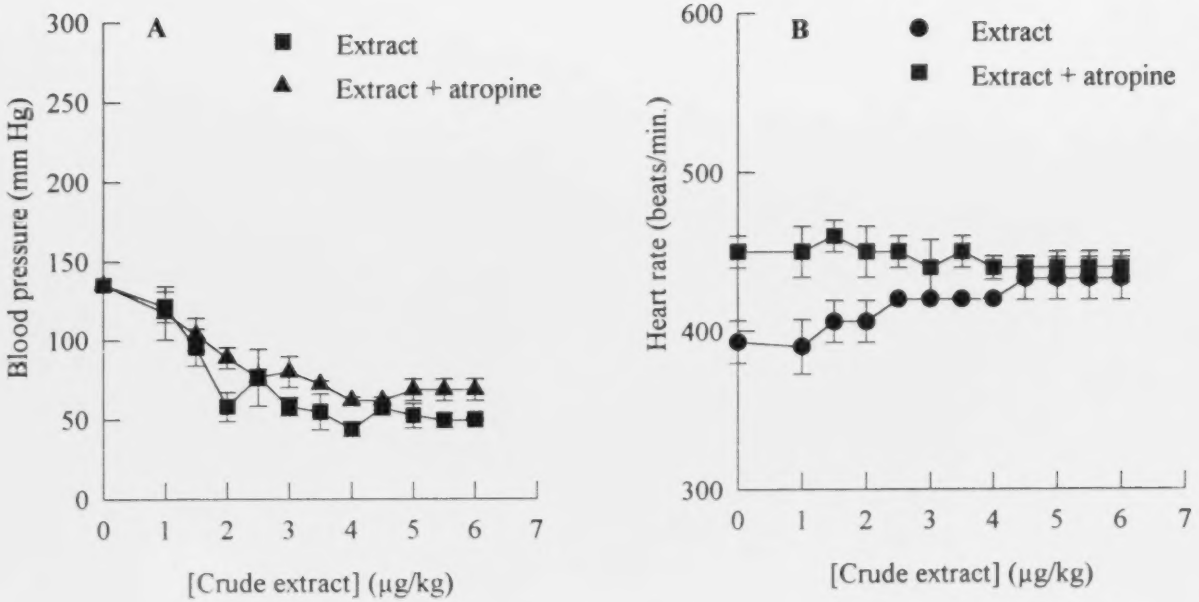


Figure 8.9 Effect of crude extract of *U. lofotensis* on arterial blood pressure (A) and heart rate (B) of anesthetized rats. Values are mean ± S.E.M. (n=3).

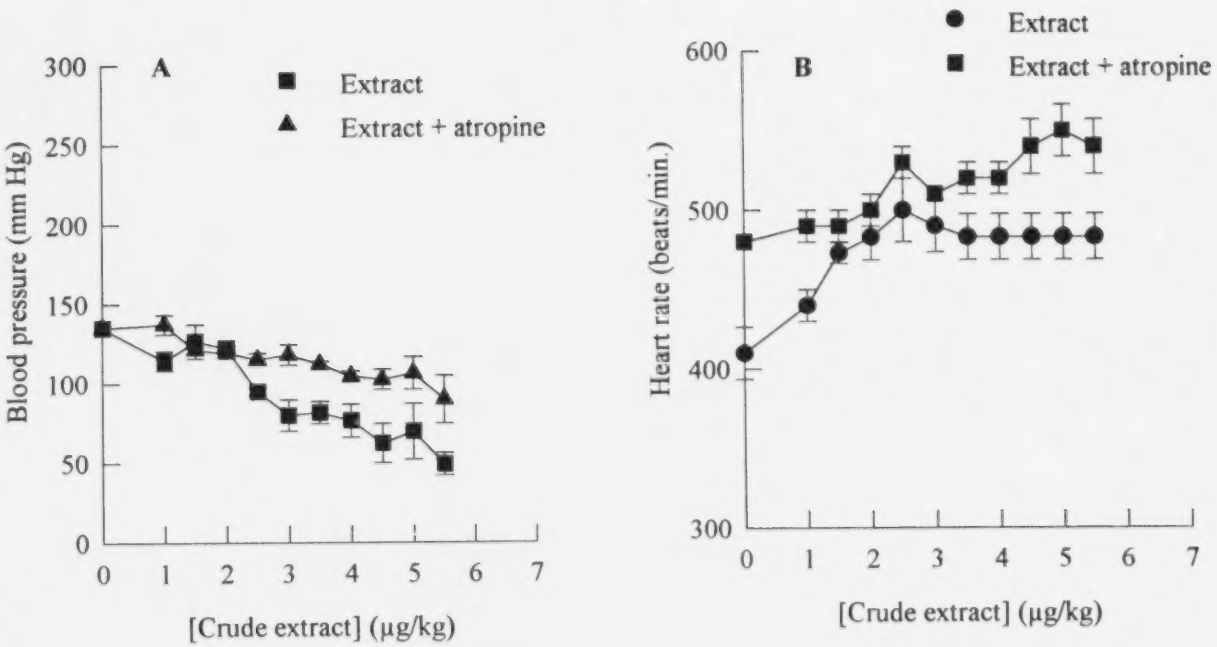


Figure 8.10 Effect of crude extract of *U. piscivora* on arterial blood pressure (A) and heart rate (B) of anesthetized rats. Values are mean ± S.E.M. (n=3).

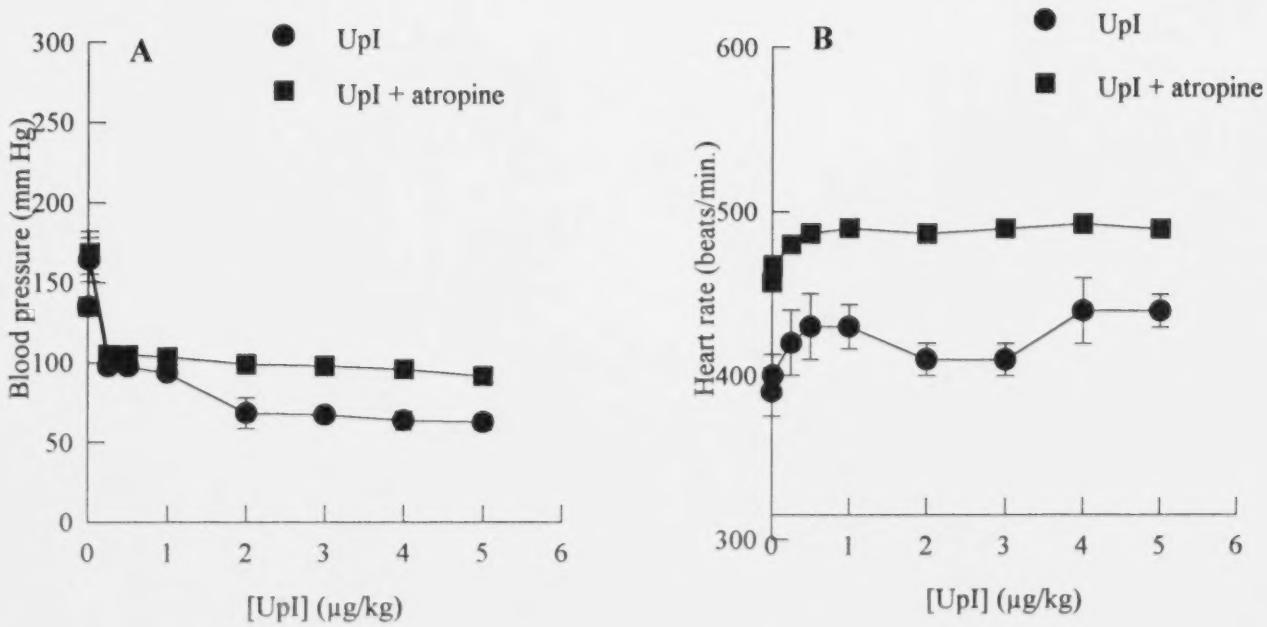


Figure 8.11 Effect of UpI on the arterial blood pressure (A) and heart rate (B) of anesthetized rats. Values are mean ± S.E.M. (n=3).

In atropinized rats, an initial increase in blood pressure was observed (Figure 8.11 B), followed by the gradual hypotension observed in all other extracts. Rats died at a much higher concentrations than in the absence of atropine. Tracings of the effect of UpI on BP and HR are shown in Figure 8.12. Note the sharp sharp decrease in BP followed by slight hypertension and then the gradual hypotension. This was typical for all the extracts tested. A summary of the postmortem findings in rats receiving extracts and UpI (Table 8.1) is illustrated. Examples of damage caused by UpI are shown in Figures 8.13.

The cause of death in each case may have been shock and internal bleeding provoked by respiratory edema, hemorrhage and extensive hemolysis, all factors which may have accounted for the severe hypotension observed.

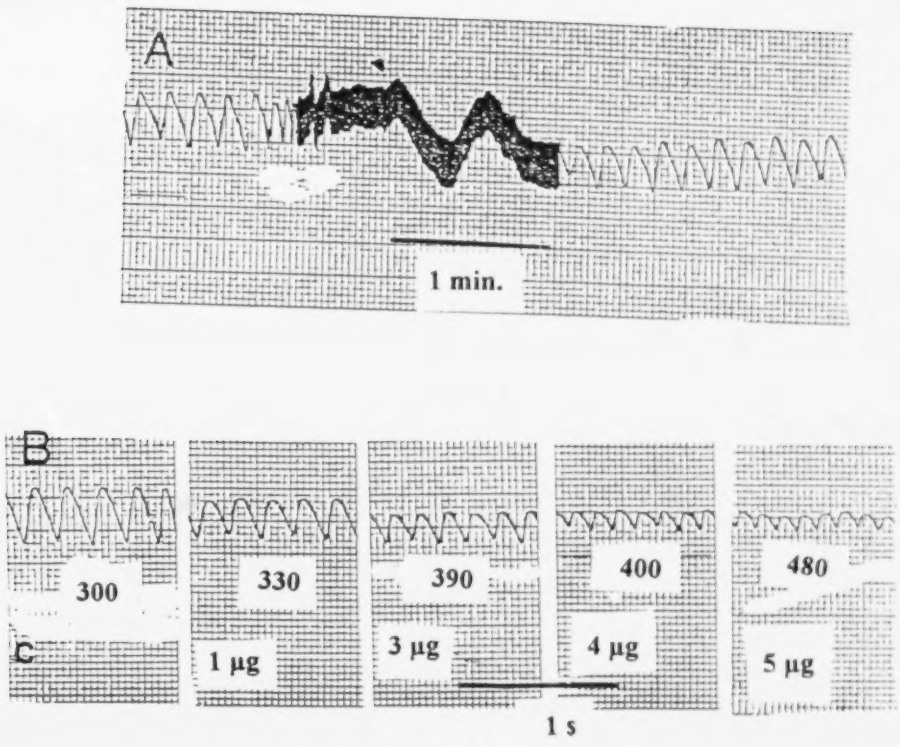


Figure 8.12 Tracings of the effect of UpI on the arterial blood pressure (A) and heart rate (B) of an anesthetized rat. Arrow indicates point of intravenous injection of UpI. Note the fall in blood pressure followed by a slight rise. (C) control heart rate.

Table 8.1. Summary of postmortem findings from pathophysiological studies

Test Product	Pathology #	# of rats	Findings	Severity
UpI	1851 R	4	necrotic skin, pulmonary mottling, hepatic lipidosis	6
UpII	1852 R	3	Normal	0
UpIII	1853 R	3	Normal	0
A. x.	1834 R	3	mild subcutaneous congestion, pulmonary congestion, hepatic lipidosis	1
U.p.	1838 R	3	subcutaneous necrosis, pulmonary mottling, hepatic lipidosis	5
U. cr.	1842 R	3	subcutaneous congestion, pulmonary mottling, hepatic lipidosis	3
P. f.	1847 R	3	subcutaneous necrosis, pulmonary mottling, hepatic lipidosis	4
U.l.	1846 R	3	subcutaneous necrosis, pulmonary mottling, hepatic lipidosis	6
S.d.	1848 R	3	subcutaneous congestion, pulmonary mottling, hepatic lipidosis	2
U. co.	1859 R	3	necrotic skin, pulmonary mottling, hepatic lipidosis	6

Key to severity score

0- No skin lesions, all organs normal
1- subcutaneous congestion, veins prominent, capillaries may be visible but not prominent
2- intense subcutaneous congestion in the caudal abdomen, veins prominent, no necrosis
3- congestion as in 1 or 2, subcutaneous necrosis
4- subcutaneous congestion, necrosis of dermis, separation of skin from body wall
5- subcutaneous congestion with edema, necrosis of dermis, abdominal wall with or without separation of skin, necrosis of skin
6- same as 5 but more extensive area involved with greater severity

Anthopleura xanthogrammica (Ax), *Urticina piscivora* (Up), *U. coriacea* (Uco), *U. crassicornis* (Ucr), *U. lofotensis* (Ul), *Stomphia didemon* (Sd), *Pachycerianthus fimbratus* (Pf), UpI, II and III, cardiac stimulatory proteins isolated from *U. piscivora.*

8.2.3 Histologic Examinations

The organ damage in rats treated with UpI was evaluated with standard histopathologic criteria. The organs most severely affected were the lungs, skin, liver and kidney. All other organs were "normal." No brain damage was detected, an indication that the toxic components of the extracts and UpI may not penetrate the blood brain barrier. There was no damage to heart tissue. The internal arrangement of organs of a UpI treated rat is shown in Figure 8.13A. Normal organ arrangement was observed.

8.2.3.1 The lungs

Gross examination of the lungs revealed severe mottling and necrosis (Figure 8.13 A). They were dark red in color and congested, *in situ*. Microscopic examination of lung tissue revealed extensive lung necrosis, hemorrhage and edema. Severe hemorrhage into the airways, vascular leakage of protein-rich fluid and rupture of the alveolar walls were common features (Figure. 8.14). UpI may exert its effect by destruction of the pulmonary vasculature, which in turn alters the flow of fluid in and out of the lungs thus resulting in edema. This may be due to direct action of UpI on the lungs or an indirect action mediated through the release of autocoids and/or catecholamines. The edemogenic effect of the extracts may or may not be due to the presence of phospholipase A activity.

Figure 8.13 (A) appearance of internal organs of rat at autopsy after injection with UpI. Arrows indicate necrotized skin and hemolysis. Note congested lungs. (B) close up appearance of the skin showing hemorrhagic spots, indicated by arrows.

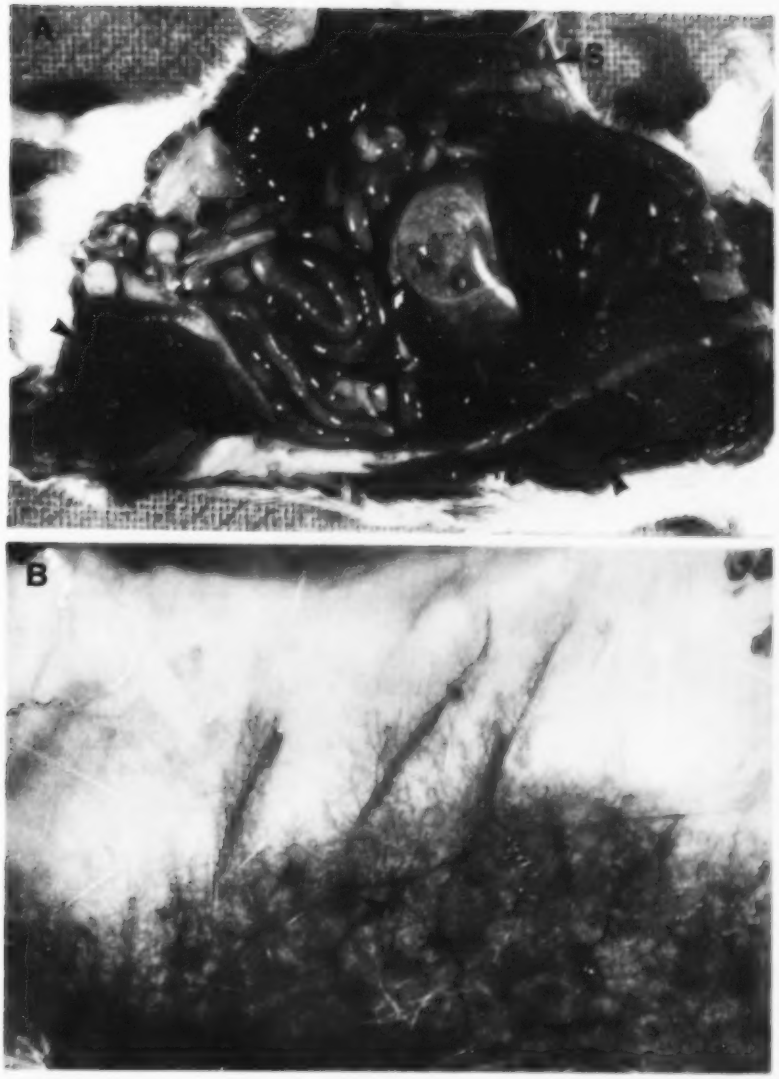
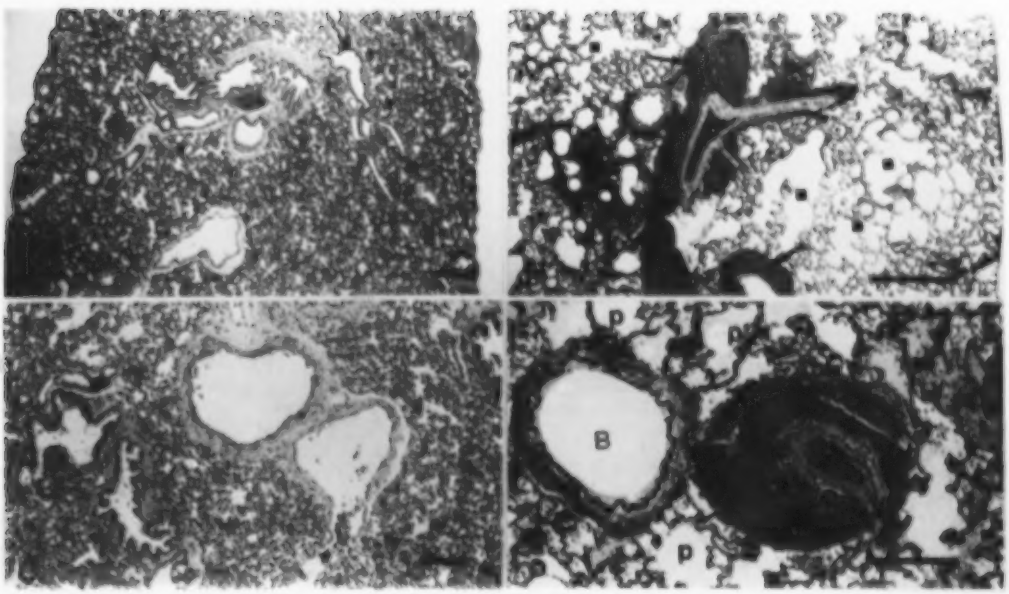

A
S
B

Figure 8.14 Histologic manifestations of UpI from *Urticina piscivora* on rat lung. (A and C) normal lung showing no sign of injury. (B and D) test lungs showing hemorrhage into the airways and lung damage. (■) indicates region of alveolar emphysema; arrows indicate hemorrhage around the pulmonary artery; (p) indicates protein rich fluid in alveoli; (B) bronchiole. For all figures (A-D), line represents 1 μm.


B
P
P
P

8.2.3.2 The skin

Damage to the skin was in the form of necrosis, hemorrhage and vascular congestion. Morphological examination of the internal organs revealed extensive hemorrhage to the visceral side of the skin together with extensive dilation of blood vessels and hemolysis (Figure 8.13 B). Microscopic examination of the skin revealed extensive necrosis of the muscle fibers (Figure 8.15).

8.2.3.3 The liver

Microscopic examination of the liver revealed coagulative necrosis of the hepatocytes and congestion of sinusoids (Figure 8.16).

8.2.3.4 The kidney

Necrotic damage caused to the liver was not as severe as in the skin or lungs, however microscopic examination revealed vascular congestion and dilation of blood vessels (Figure 8. 17).

Figure 8.15 Histologic manifestations of UpI on skin of rat. (A) Section through normal rat skin showing no sign of injury and regular arrangement of cutaneous trunci (cm). (B) indicates section through skin of test animal showing hemorrhage into the deeper dermis (●) exposing veins; arrows indicate interstitial edema and separation of muscle fibers. Note the disintegration of cutaneous trunci. (E), epidermis; (spd), superficial dermis; (dd) deeper dermis; (sbd) subcutaneous dermis; (cm), cutaneous trunci or muscle; (v), blood vessel. Line represents 1 μm.

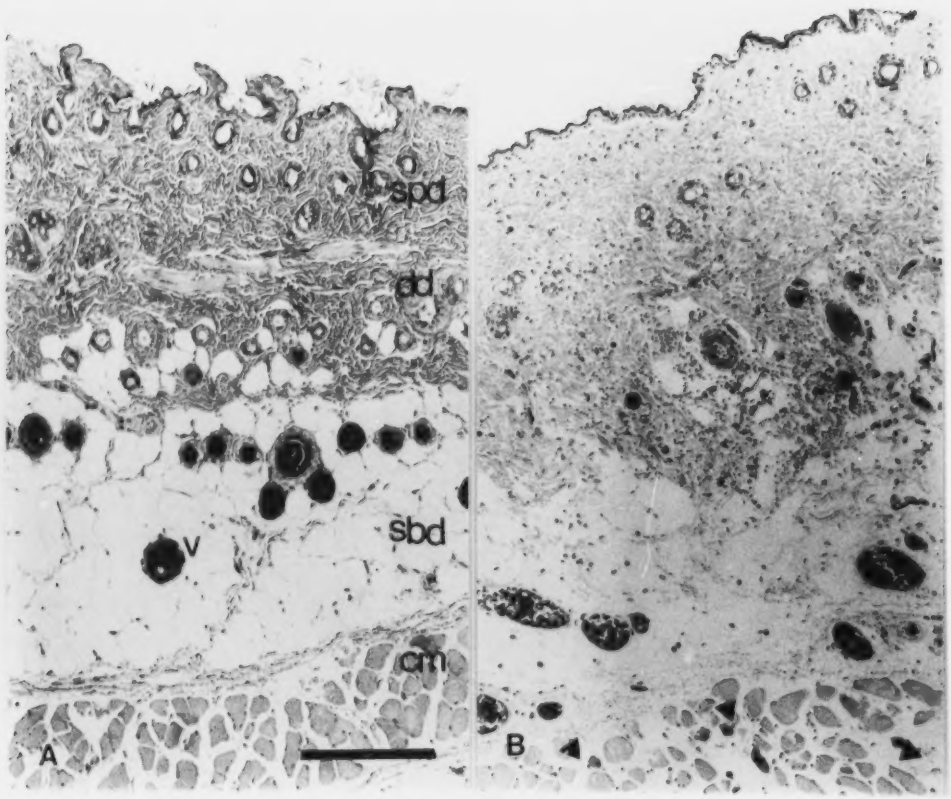

spd
dd
v
sbd
cm
A
B

Figure 8.16 Histologic manifestations of UpI on rat liver. (B) liver of normal rat showing no sign of injury and (A) liver of test animal showing signs of sinusoid congestion and necrosis as indicated by arrows.

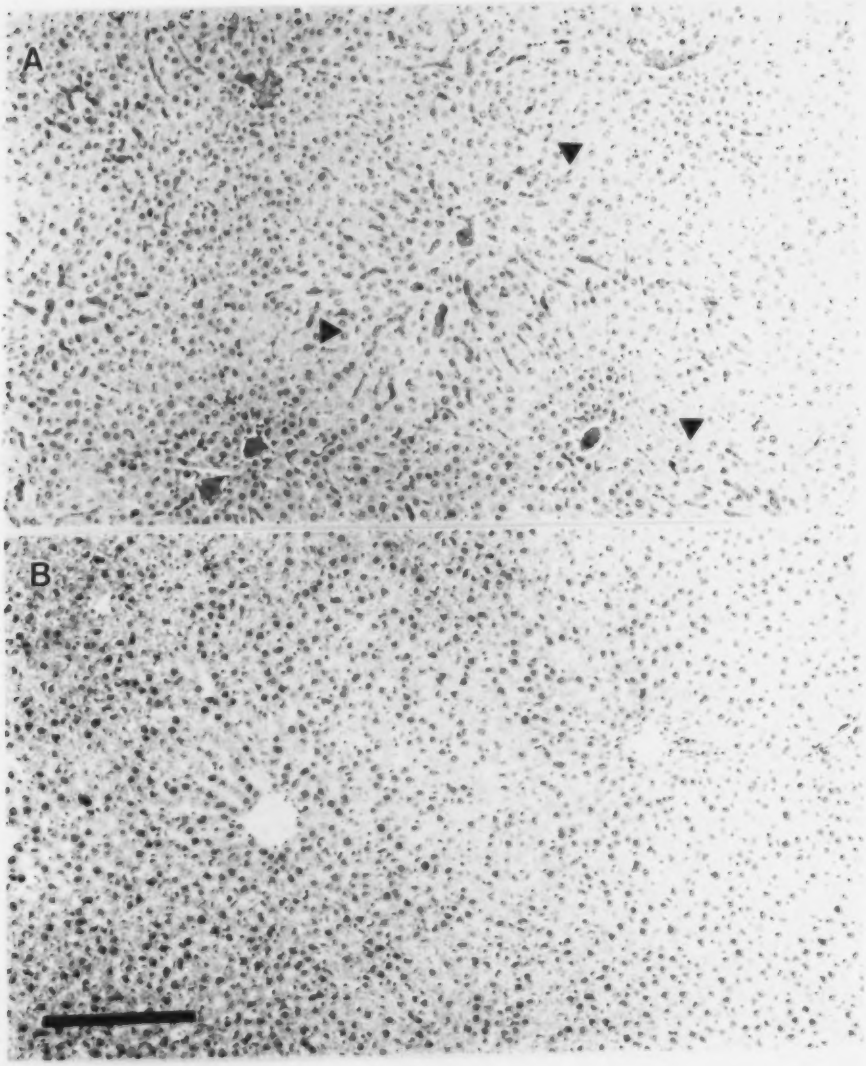

A
B

Figure 8.17 Histologic manifestations of UpI on kidney of rat treated with UpI. (A) normal kidney with no signs of injury and (B) section through test kidney showing vascular congestion and dilated blood vessels indicated by arrows.

A
B

8.3 Discussion and conclusions

All crude extracts tested, together with UpI, were found to be toxic to anesthetized rats. They produced a transient fall in arterial blood pressure followed by progressive bradycardia and finally respiratory arrest. Urethane produced slight shivering on intraperitoneal administration and may have had a synergistic effect, thus potentiating the hypotensive action of the test compounds. This may be due to its α_2 receptor blocking effect or the release of norepinephrine. Although, urethane has been reported to prevent the appearance of pulmonary edema induced by scorpion toxins in rats,[13] it was not our intention to determine the effect of anesthesia on the effect of the extracts or proteins.

Hypotension immediately after intravenous injection of extracts and proteins may be due to loss of sympathetic tone. There was no appreciable cardiovascular effect except for bradycardia which was always immediately accompanied by respiratory arrest. These responses are similar to those observed with snake bites, venom and toxins such as taipoxin and bungarotoxins.[14] Circulatory shock with internal hemorrhage is a frequent cause of death after viper bites.[15] Such effects have not been reported for any of the known sea anemone toxins evaluated.

Several hemorrhagic principles have been isolated and characterized from snakes,[16,17] sea nettles[18] and jellyfish.[19] Some snake venoms are known to produce sharply demarcated hemorrhagic spots that spread throughout the dermis and muscular layers,[20] similar to those observed (Figure 8.13B) in this study. Oshaka[20] has correlated the size of

the spot to the concentration of the venom. The effects observed with UpI are also similar to those induced by the β-bungarotoxins from the snake venom of *Bungarus multicintus*[14] and *Trimeresurus gramineus*.[16] Limited similarity between UpI and the β-bungarotoxins has been established.[21] Equinatoxin II (EqT II), the only sea anemone toxin to have been studied extensively, exerts its effect by inducing pulmonary edema, cardiac arrhythmias and hypotension.[12,22,23]

Unlike UpI, EqT II is cardiotoxic at high concentrations[9,24] and induces a transient negative inotropic action at low concentrations on atrial tissues.[22] It produces only a negative inotropic effect on ventricles,[12] an action similar to that observed with the crude extracts (Figure 4.2). The edemogenic activity of marine toxins and venom has been attributed to the presence of phospholipases, that induce degranulation of mast cells, hemorrhagic activity causing vascular damage[25] and vascular permeability associated with the release of some peptides.[17,19]

Several Cnidarian toxins have been shown to have toxic effects.[14,26,27] The cause of death by venom and toxins from some of these organisms is somewhat different from these observations with the sea anemone extracts and UpI. The sea nettle venom *Chrysaora quinquecirrha* exerts its effect by inducing severe hepatic necrosis[18] and cardiotoxicity[28] while palytoxin from *Palythoa sp.*, the most potent toxin known, elicits its contractile effect directly on smooth muscle and not via muscarinic receptors.[29] Other causes of death include renal failure after jellyfish stings in humans caused by either toxic or ischemic injury.[30]

A typical case report by Bengston *et al.*,[31] was of a 4 year old boy, who died within 40 min. of being stung by a specimen of the jellyfish *Chiropsalmus quadrumanus*. An autopsy completed 19 hours after death, revealed whitish foam in the trachea, lesions along the thorax and eruptions on the arms. Internal examination revealed a normal organ arrangement and frothy fluid within the larynx, trachea, and bronchi. The lung weighed 530 g, as compared to the normal 211 g and appeared purple and congested. Examination of the liver, spleen and kidneys revealed acute congestion. The immediate cause of death of the boy was given as cardiac shock, hypotension and pulmonary edema following envenomation. Such reactions are quite common in Australia where over 60 fatalities have been reported.[30] A similar effect has not been ruled out for toxins isolated from dinoflagellates.[32]

In conclusion, this Chapter shows that both crude extracts and UpI induced toxic effects in anesthetized rats. Also, it is quite clear from the results that UpI is one of the proteins within the extract eliciting the toxic effect. UpII and UpIII can be considered as being much less toxic.

At present, the actual mechanism of toxic action of the extracts and UpI cannot be commented upon. Additional studies of hemodyanamic effects of the extracts and UpI are required to define their effects. However, the present data, show that intravenous injection of the purified toxin UpI from *U. piscivora* induces the same toxic action as the crude extract, but at a much lower concentration. Death resulting from the test extracts and UpI appear, to be related mainly to respiratory, dermal and slight renal and hepatic

injury, but not to cardiac or cerebral injury. It can be concluded that death was due to hypotension, pulmonary edema and vascular collapse.

Bibliography

1. Friese, U. E. (1972) *Sea Anemones*. pp. 1-128, T F. H. Publications Inc. Ltd. Hong Kong.

2. Massmanian, A., Cavero, V., Bosca, R. and Rodellas, C. (1988) Sea anemone dermatitis. *Contact Dermatitis* **18**, 169-189.

3. Nicholls, D. (1995) Sea anemone sting while SCUBA diving. *N. Z. Med. J.* **105**, 245-246.

4. Wunderer, G., Machleidt, W. and Watcher, E. (1976) Toxin II from *Anemonia sulcata* - the first sequence of a coelenterate toxin. *Hoppe-Sey. Z. Physiol. Chem.* **357**, 239-240.

5. Halstead, B. W. (1980) *Dangerous Marine Animals*. pp. 57-58, Cornell Maritime Press, Centreville, Maryland.

6. Alsen, C., Beress, L. and Tesseraux, I. (1978) Toxicities of sea anemone (*Anemonia sulcata*) polypeptides in mammals. *Toxicon* **16**, 561-566.

7. Macek, P. and Lebez, D. (1988) Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L. *Toxicon* **26**, 441-451.

8. Wunderer, G., Fritz, H., Watcher, E. and Machleidt, W. (1976) Amino acid sequence of a coelenterate toxin: Toxin II from *Anemonia sulcata*. *Biochem.* **68**, 193-198.

9. Ho, C. L., Ko, J. L., Lue, H. M., Lee, C. Y. and Ferlan, I. (1987) Effects of equinatoxin on the guinea pig atrium. *Toxicon* **25**, 659-664.

10. Kem, W. R. and Dunn, B. M. (1988) Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon* **26**, 997-1008.

11. Sket, D., Draslar, K., Ferlan, I. and Lebez, D. (1974) Equinatoxin, a lethal protein from *Actinia equina*. II. Pathophysiological action. *Toxicon* **12**, 63-68.

12. Bunc, M., Frangez, R., Horvat, I., Turk, T. and Suput, D. (1994) Effects of equinatoxins *in vivo*. Possible role of degranulation of thrombocytes and granulocytes. *Toxins and Exocytosis. Ann. N. Y. Acad. Sc.* **710**, 162-167.

13. Freire-Maia, L., Almeida, H. O., Cunha-Melo, J. R., Azevedo, A. D. and Barroso, J. (1978) Mechanism of pulmonary edema induced by intravenous injection of scorpion toxin in the rat. *Agents and Action* **8**, 113-118.

14. Lee, C. Y., Lin, W., Chen, Y. M. and Lee, S. (1987) On the causes of acute death produced by animal venom and toxins. In: *Progress in Venom and Toxin Research: Proceedings of the First Asia-Pacific Congress on Animal, Plant and Microbial toxins* (Gopalakrishnakone, P. and Tan, C. K. eds.) pp. 3-15, University of Singapore Press. Singapore.

15. Lee, C. Y. and Lee, S. Y. (1979) Cardiovascular effects of snake venom. In: *Handbook of Experimental Pharmacy.* (Lee, C. Y. ed.) pp. 547-590, Springer, New York.

16. Huang, T., Chang, J. and Ouyang, C. (1984) Characterization of hemorrhagic principles from *Trimeresurus gramineus* snake venom. *Toxicon* **22**, 45-52.

17. Teng, C., Wang, J., Peng, H. and Ouyang, C. (1989) Edema-producing proteins isolated from *Trimeresurus mucrosquamatus* snake venom. *Toxicon* **27**, 899-905.

18. Muhvich, K. H., Sengottuvelu, S., Manson, P. N., Myers, R. A. M., Burnett, J. W. and Marzella, L. (1991) Pathophysiology of sea nettle (*Chrysaora quinquecirrha*), envenomation in a rat model and the effects of hyperbaric oxygen and verapamil treatment. *Toxicon* **29**, 857-866.

19. Azila, N., Siao, F. and Othman, I. (1991) Hemolytic, edema and hemorrhage inducing activities of tentacular extract of the blubber jellyfish (*Catostylus mosaicus*). *Comp. Biochem. Physiol.* **99C**, 153-156.

20. Ohsaka, A. (1972) Hemorrhagic, necrotizing and edema-forming effects of snake venom. In: *Handbook of Experimental Pharmacology: Snake Venom.* (Lee, C. Y. ed.) pp. 481-546, Springer, Heidelberg.

21. Cline, E. I., Wolowyk, M. W., Wiebe, L. I., George, R. and Samuel, J. (1995) Isolation and characterization of a novel cardiac stimulatory and hemolytic protein from the sea anemone *Urticina piscivora* (Sebens and Laakso). *Pharm. Sci.* **1**, 155-162.

22. Budihna, M., Suput, D. and Macek, P. (1990) Cardiac arrhythmia and negative inotropic effect induced by equinatoxin II. *Int. Conf. IEEE Eng. Med. Biol. Soc.* **12**, 656-657.

23. Lafranconi, W. M., Ferlan, I., Russell, F. E. and Huxtable, R. J. (1984) The action of equinatoxin, a peptide from the venom of the sea anemone *Actinia equina* on the isolated lung. *Toxicon* **22**, 347-352.

24. Lee, C. Y., Lee, S. Y., Chen, Y. M., Lue, H. M. and Ferlan, I. (1986) Coronary vasospasm as the primary cause of death due to equinatoxin, a toxic protein from *Actinia equina*. *Toxicon* **23**, 586.

25. Azila, N. and Othman, I. (1990) Hemolytic and phospholipase A activities of the tentacle extract of *Castostylus mosaicus*. *Biochem. Int.* **20**, 291-299.

26. Ito, K., Urakawa, N. and Koike, H. (1982) Cardiovascular toxicity of palytoxin in anesthetized dogs. *Arch. Int. Pharmacodyn. Ther.* **258**, 146-154.

27. Lin, W., Lee, C. Y., Burnett, J. W. (1988) Effect of sea nettle (*Chrysaora quinquecirrha*) venom on isolated rat aorta. *Toxicon* **30**, 1209-1212.

28. Burnett, J. W. and Calton, G. J. (1977) The chemistry and toxicology of some venomous pelagic coelenterates. *Toxicon* **15**, 177-196.

29. Ito, K., Karaki, H. and Urakawa, N. (1977) The mode of contractile action of palytoxin on vascular smooth muscle. *Eur. J. Pharmacol.* **46**, 9-14.

30. Lumley, J., Williamson, J. A., Fenner, P. J., Burnett, J. W. and Colquhoun, D. M. (1988) Fatal envenomation by *Chironex fleckeri*, the north Australian box jellyfish: the continuing search for lethal mechanisms. *Med. J. Aust.* **148**, 527-534.

31. Bengston, K., Nichols, M. M., Schnadig, V. and Ellis, M. D. E. (1991) Sudden death in a child following jellyfish envenomation by *Chiropsalmus quadrumanus*. *J. Am. Med. Assoc.* **266**, 1404-1406.

32. Terao, K., Ito, E., Murakami, M. and Yamaguchi, K. (1989) Histopathological studies on experimental marine toxin poisoning-III. Morphological changes in liver and thymus of male ICR mice induced by goniodomin A, isolated from the dinoflagellate *Goniodoma pseudogoniaulax*. *Toxicon* **27**, 269-271.

IX. GENERAL DISCUSSION AND CONCLUSIONS

9.1 Discussion and conclusions

This research study was carried out from the point of view of searching for biomedically useful leads with potential therapeutic implications. The pharmacological investigation of the active agents described in the thesis provides a better knowledge of bioacive substances from a marine source, sea anemone. The research provides information that could be used in the effective treatment of human and animal intoxication due to marine venom. Today, there is increased utilization of marine products as food materials (for example fish and algae), in which substances formed by a number of diverse organisms may be included along with the food. This poses real danger of mass intoxication that may be acute. A better knowledge of marine pharmacology thus enables the preparation of better foods.

In this work, an attempt has been made to provide information relating to bioactive polypeptides and proteins derived from sea anemones. A further attempt has been made to consider them in terms of their biomedical, pharmacological and toxicological potential.

Eleven species of sea anemones belonging to four known families were collected from the west coast of Canada. The extraction procedure utilized was effective in yielding extracts with different biological activities. Extracts were assayed for cardiotonic, hemolytic, cytotoxic and antifungal activities. While some extracts possessed one or more activities, they were all devoid of antifungal activity. Of all the sea anemone species

examined, only the crude extract of *Pachycerianthus fimbratus* was devoid of cardiotonic and cytolytic activities. The fact that the extract of *P. fimbratus* showed moderate cytotoxic acticity yet was devoid of proteins indicates that perphaps only nonproteinaceous toxins are present in this species.

As stated earlier, the initial intent of this study was to identify cardiotonic agents from sea anemones. All previous studies have indicated that the cardiotonic, cytotoxic and hemolytic agents from sea anemones were either polypeptides or proteins. However, this research was approached assuming that either primary and or secondary metabolites could be responsible for any of the biological activities. Before the active component could be isolated, it was necessary to identify a tissue sensitive enough to be used for the as bioassay for cardiac activity. The atria of rat and guinea pig were very sensitive to the extracts while the ventricles were not. In most instances the ventricles elicited negative inotropic responses. Such a negative inotropic response has been associated with equinatoxin II (EqT II) from the sea anemone *Actinia equina*.[1,2] Rat atria proved to be a cheap and highly sensitive assay for guiding successful isolation and purification of active cardiotonic agents.

Each of the eleven species collected was assayed for cardiotonic activity and eight of them elicited varying degrees of positive inotropic activity. While *Metridium senile*, *Urticina coriacea* and *P. fimbratus* were inactive, *Epiactis prolifera* was cardiotoxic at high doses. The results presented are consistent with preliminary investigations of extracts from the west coast of the United States which were first reported to have cardiotonic activity by Shibata and his co-workers.[3]

Hemolysis assays have been reported to be the simplest, most sensitive procedure for detecting cytolysins. Hemolysis can be detected by simple visual inspection.[4] It has been used by many researchers to isolate and characterize cytolysins from over twenty species of sea anemones.[5,6] Of the eight extracts assayed for this activity, only *P. fimbratus* was inactive. Bernheimer and Avigad[7,8] first reported variations in the sensitivity of erythrocytes to sea anemone extracts and cytolysins. Comparisons of *Stichodactyla helianthus* and *Metridium senile* showed that species differences in sensitivity were often quite large. Some differences in sensitivity of erythrocytes were observed for some of the extracts used in the assay as illustrated below (Table 9.1).

Table 9.1 Sensitivity of erythrocytes from three mammalian species to extracts of sea anemones

Sea anemone	HC_{50} (μg/ml)		
	Rat	Guinea pig	Dog
A. xanthogrammica	9.2	8.3	10.5
M. senile	9.5	9.3	5.0
P. fimbratus	IN	IN	IN
S. didemon	5.0	7.0	8.5
U. coriacea	6.5	7.5	4.7
U. crassicornis	3.0	7.2	7.6
U. lofotensis	9.8	8.0	6.3
U. piscivora	2.3	6.2	4.3

HC_{50} concentration (μg/ml) at which 50 % hemolysis was achieved, IN indicates inactive.

Six of the eleven sea anemones showed *in vitro* cytotoxicity against the three cell lines used. Extracts of *U. piscivora* and *U. coriacea* were undoubtedly the most potent. The others showed moderate or no toxic activity. There was no correlation between the activity of the extracts and the sensitivity of cell lines, however HEL 299 seemed to be the most sensitive to most extracts.

All extracts were devoid of antifungal activity, a result similar to those reported by Mahnir *et al.*,[9] who showed that twenty-two species of anemones from the North Pacific were devoid of both antifungal and antibacterial activity.

One conclusion that can be drawn from the preliminary screening reported is that sea anemones prevalent along the west coast of Canada possess biological activities similar to those of sea anemones from United States,[3] Israel,[10] North Adriatic coast,[11] North Pacific,[9] Japan[12,13] and the Atlantic ocean.[14,15]

Of the extracts assayed for cardiotonic activity, the most active were *Anthopleura elegantissima* and *A. xanthogrammica*, followed by *Urticina piscivora*. However since the cardiotonic agents of *A. elegantissima* and *A. xanthogrammica* have been fully characterized and pharmacologically evaluated,[16-19] *U. piscivora* was the next obvious choice for further investigation.

Successful isolation and purification of the active cardiotonic agents from *U. piscivora* was achieved by a combination of chromatographic methods of separation. They included size exclusion, cation exchange chromatography, and analytical and preparative reverse phase HPLC. Similar methods have been widely employed in the

successful separation and purification of cardiotonic and or cytolytic polypeptides and proteins from sea anemones.[5,6] For the purification of tenebrosin A (TN-A) from *Actinia tenebrosa*, cation exchange HPLC was employed,[20] while successful separation of the isotoxins magnificalysin I and II from *Heteractis magnifica*, could only be achieved by hydrophobic interaction chromatography.[21]

Three active cardiotonic proteins were subsequently isolated and purified from *U. piscivora*. Their molecular weights were UpI (28 kDa) and UpII and III (19 kDa). All three proteins were eluted in the same fraction as trypsin inhibitor (mol. wt. 20 kDa; Ve 475 ml) on gel filtration chromatography. However, some sea anemone cytolysins have been reported to interact with gel support and elute in regions of molecular weights far less than their actual molecular mass. Such an anomalous elution pattern has been observed for cytolysins from *Stichodactyla helianthus*[22] and anthopleurin B from *A. xanthogrammica*.[19] The latter behaved anomalously on sephadex columns and eluted like a peptide with a molecular weight of 2,000 Da while its actual molecular weight was 5,267 Da.[19] Toxins from *Aiptasia pallida* thought to have molecular weights of 30 kDa eluted in the same fraction as cytochrome C (10-15 kDa).[23]

It is worth noting that during the first step of separation (gel filtration chromatography), some of the fractions, after freeze drying, became very light and powdery, so that they became easily airborne during handling. Whenever this occurred, it caused conjunctivitis and severe pulmonary congestion. However, once the fractions were placed in solution they did not pose any health problems.

The three cardiotonic proteins purified were designated UpI, UpII and UpIII. Although all three proteins occurred within a single fraction (fraction C) of the gel chromatographic separation, resolution by cation exchange was able to separate them into two fractions, one containing UpI and the other both UpII and III. The purity of all three proteins was confirmed by analytical reverse phase HPLC, with retention times of UpI (23 min.), UpII (17.1 min.) and UpIII (17.8 min.).

The nomenclature adopted was based on the first letter of the genus and species respectively, and numbering them based on which was isolated first. In 1986, Bernheimer[24] proposed a nomenclature for sea anemone bioactive polypeptides and proteins, based on the already established nomenclature used for cytolysins from bacteria.[25] In lieu of their cytolytic nature, lysin was added to the end of the proposed name derived either from the genus or species of the anemone. For example, epiactin from *Epiacitis prolifera* becomes epiactolysin. If one were to adopt this nomenclature on the assumption that all three proteins were lytic in nature, they would be called, Urticinalysin I, II and III or piscivoralysin I, II and III or A, B and C as the case may be. In a situation where the lytic property of the proteins were de-emphasized, then they would be designated piscivorotoxin I, II and III or A, B and C respectively. What makes this nomenclature questionable, is that Bernheimer[24] assumed that all proteins isolated from sea anemones with high molecular weights (10-30 kDa) would be cytolytic. However it is quite obvious from the previous chapters that UpII and III (19 kDa) were neither cytolytic nor very toxic in nature and so they are not ideal candidates for this proposed nomenclature, even though their molecular weight qualifies them as cytolysins.

Considerable similarities and differences have been revealed in the amino acid sequence of cytolysins.[6] To date all but one of the known cytolysins are basic, with a large number of basic amino acids.[5] The most abundant amino acids found in cytolysins are glycine, alanine, leucine, valine and threonine. The most common aromatic amino acids are tyrosine and phenylalanine. Most cytolysins, however, lack cysteine except for kentin which has four,[14] epiactin B with one[26] and *Condylactis* cytolysin with fourteen residues.[6] UpI was found to be basic, consisting of the usual amino acids common to cytolysins. It has ten cysteine, arginine and tyrosine residues, eight phenylalanine, three methionine and twenty-one lysine residues. UpII and III on the other hand seemed to be highly conserved, containing very similar amino acid compositions (Table 6.1) and partial sequences (Table 6.2). Arginine, glycine and cysteine were the most abundant residues, consisting of about 17 % of the total residues respectively. While UpIII was devoid of methionine, UpII had just one residue and both were devoid of phenylalanine. UpII and UpIII were considered neutral based on their pI's.

Tanaka *et al.*[17] and Reimer *et al.*[19] first sequenced the heart stimulants anthopleurin A and B, respectively, by manual Edman degradation. Since the advent of automated sequencers, the partial amino acid sequences of over fifteen cytolysins have been determined.[5,6] So far the complete primary structures of only two sea anemone cytolysins have been elucidated; those of Sh III from *Stoichactis helianthus*[27] and tenebrosin C from *Actinia tenebrosa*.[28] Automated amino acid sequencing of the intact protein was used to determine the partial sequences of intact UpI, UpII and UpIII. While UpII and UpIII

exhibited approximately 50 % homology, they showed very little similarity to UpI. A comparison of the partial sequences of UpI, II and III is illustrated (Table 9.2).

Table 9.2 Comparison of the partial N-terminal sequences of UpI, UpII and UpIII from *U. piscivora*

UpI									DENENL		YGP	-	NE		NKA		K		AKDLTAGASY
UpII	AT	D	K	W	ND		CGHVTALC		EQK	-	GF				NKA		T		C
UpIII		D	D	D	W	DE		CGHVTAL	L		EGQ	Q	GR		NKA		A		C
		*		*			*	***	***	+	*+		*						*

Boxed letters indicate identical residues between UpI, UpII and UpIII

(-) gaps introduced by the program to give optimal alignment

(*) identical residues between UpII and UpIII

(+) identical residues between UpI and at least one other sequence

The purity of all three proteins was further established by amino acid analysis, sequencing and electrofocusing. Based on their resolutions by SDS-PAGE in reducing and non-reducing conditions, all three proteins appeared to exist as single polypeptide chains consisting of approximately 254 amino acids (UpI), 188 (UpII) and 178 (UpIII), respectively. UpII and UpIII however had different molecular weights when resolved in the presence and absence of β-mercaptoethanol and occured as single bands each. This may indicate the presence of sub-units with identical molecular weights. Once the purity

of the proteins was established, it was necessary to evaluate their biological activities to determine if they were responsible either singly or collectively for the activities observed with the crude extracts from which they came.

The establishment of criteria for the determination of comparative parameters of pharmacological activity of drug molecules is a relatively discretionary process, based on the investigator's knowledge or experience with the class of drug. The basis of determining criteria may include the chemical class(es), the observed pharmacological effects and the mechanism of action of the drugs if known.

Spedding and Berg[29] proposed that experimental results should provide a quantitative estimation of relative potency, with index for potency being the negative log of concentration causing half the maximal effect. Once the effect of each protein was evaluated relative to the known positive inotrope isoproterenol (INA), it was necessary to compare the potencies of each. The approach used in this thesis was a quantitative one in which the proteins were classified with their EC_{50}'s and dividing the EC_{50} value of the least potent protein by the EC_{50} value of each protein and INA respectively. The figure arrived at was the relative potency (RP) of that compound. The least potent compound was given a value of one. Table 9.3 shows the RP and EC_{50} values of all three cardiotonic proteins from *U. piscivora*. Relative to INA, the most potent protein was UpI (RP = 864), although its RP value was three fold less than INA. The least potent was UpIII (RP = 1.0). In terms of relative potencies, the degree of positive inotropic activity decreased in the order, INA > UpI > UpII > UpIII for rat left atria. Whether the same will hold true for other mammalian heart tissues will have to be determined. Based on their

EC_{50} values, the cardiotonic action of UpI is comparable to that of the potent anthopleurin A (EC_{50} 4.4 x 10^{-9} M).[17]

Table 9.3 Comparison of the relative potencies of the cardiac stimulatory proteins UpI, UpII and UpIII from *U. piscivora*

Protein	EC_{50} (M)	± sem	n	RP
UpI	8.1 x 10^{-9}	2.7	3	864
UpII	10^{-7}	3.1	3	70
UpIII	7 x 10^{-6}	1.7	3	1.0
INA	3 x 10^{-9}	2.2	9	2333

EC_{50} is the concentration (M) of the protein and INA that elicits a 50 % increase in contraction of the rat left atria
RP indicates relative potency
n is the number of animals
sem indicates standard error of mean

A comparison of all three dose response curves in relation to INA is illustrated together with the maximum responses achieved for each in Figure 9.1. The mechanism of action of the three cardiotonic proteins cannot be commented upon at present since it was not evaluated. Further pharmacological evaluation is required before this can be determined. However several researchers have established that sea anemone polypeptides

alter the function of the sodium channel in excitable tissues by delaying the inactivation process.[4] Their potential therapeutic property is their ability to selectively stimulate cardiac contractility in intact mammals without affecting neuromuscular function.[30] At this time, it is still not clear how the polypeptides actually increase cardiac contractility, although their direct effect is to stabilize the open state of the sodium channel, thereby enhancing influx during the heart beat.[4] Whether or not UpI, II and III exert cardiac contractility in a similar manner to these low molecular polypeptides will still have to be determined.

The main property of sea anemone cytolysins is their ability to form discrete pores in the membranes which lead to total cell lysis. The hemolytic action of the crude extracts, their molecular weights and pulmonary congestion evoked in the laboratory by some fractions, made it necessary to evaluate all three proteins from *U. piscivora* for potential cytolytic activity. While UpI proved to be a potent hemolysin on five different erythrocytes, UpII and UpIII were devoid of hemolytic activity. It can thus be concluded that UpII and III were not the proteins responsible for the hemolytic action of the crude extract, while UpI may be one of the proteins eliciting this activity.

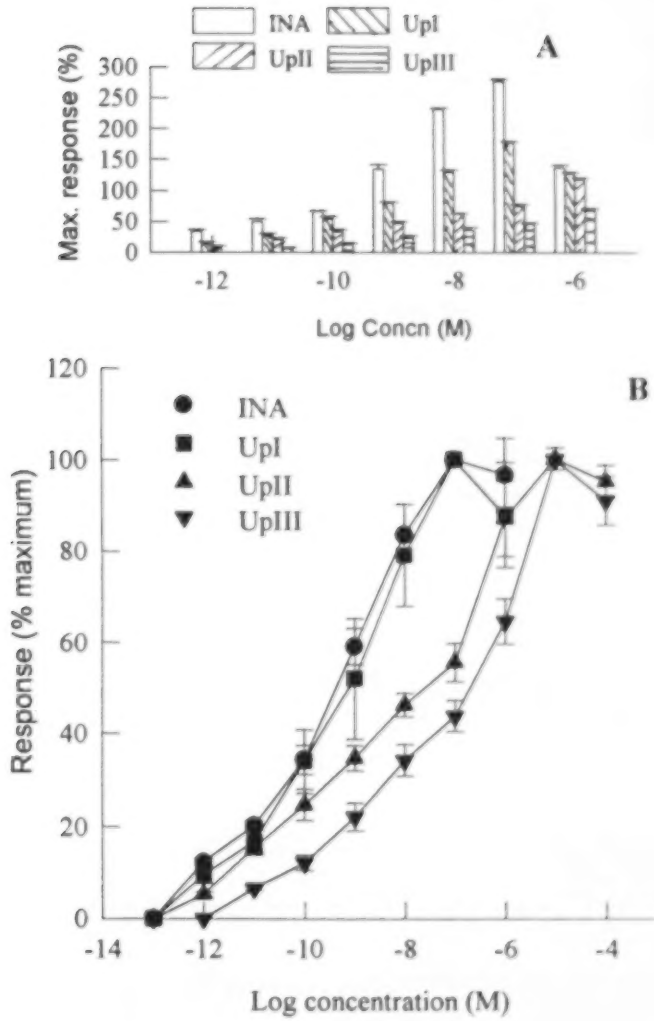


Figure 9.1 Comparison of the maximum responses (A) and dose response (B) of UpI, II and III with isoproterenol (INA). Values are mean ± S.E.M. (n=3) except for INA where (n=9).

Based on the sigmoidal time course of hemolysis,[6] the following mechanism of action has been proposed.[4-6] In the prolytic step of hemolysis a direct lytic factor (the protein cytolysin) binds to the cell membrane, enhancing a phospholipase attack on the membrane phospholipids. The lytic phase follows, where hydrolysis of the phospholipids occurs followed finally by an asymptotic phase where the remaining cells are lysed. A different mechanism of action has been suggested for those sea anemone cytolysins which lack phospholipase A activity. Macek *et al.*[31] suggested a colloid-osmotic shock, mediated by the formation of pores as their mechanism of action. They based this conclusion on microscopic inspection of the lytic events of red blood cells. Whether UpI acts via any of these mechanisms still has to be determined.

A relationship is said to exist between pI's of sea anemone cytolysins and their hemolytic activities. The more acidic the cytolysin, the greater its hemolytic action as compared to basic ones.[21] However, more data are needed to ascertain whether this applies to UpI as well.

UpI as a cytotoxic agent was not as potent as the already established cytotoxic equinatoxin (EqT II), which was active as concentrations as low as 2 nM.[32-34] UpII and UpIII were not assayed for cytotoxic activity.

The use of scanning electron microscopy to investigate and observe structural damage to erythrocyte membranes brought about by anemone toxins is rare. However using the transmission electron microscope, researchers have shown that metridiolysin from *Metridium senile* produces ring like structures on the membranes of sheep

erythrocytes.[35] Morphological damage to the membranes has been well documented for bacterial cytolysins.[36,37] Use of the scanning electron microscope will provide the much needed information on the structural damage caused to erythrocyte membranes by new and already known characterized cytolysins.

The complexity of Cnidarian venoms has been well documented. These compounds are reported to contribute to the animal's defense and digestion.[38-40] Stings by some of these species do cause immediate pain with considerable damage to the tissues surrounding the sites of envenomation.[40,41] The most common of these effects is inflammation followed by erythematous eruption and hemorrhage.[41] These effects, together with the edema producing action of Cnidarian venom and extracts, have been associated with the activity of phospholipase A present in the extracts.[42] Histamine release and alteration in mast cell morphology have been associated with the venom of *Physalia physalis*.[43]

Pathophysiological investigation revealed that UpI elicited the same toxic effects observed for the crude extracts. Based on microscopic examination, it can be concluded that UpI is one of the toxic constituents of *U. piscivora*. Like the crude extract, UpI produced respiratory failure, lung hemorrhage, circulatory failure due to hemolysis and skin necrosis, all common features observed from bites of snakes belonging to the families Elapidae, Crotalidae and Viperidae.[44] UpII and UpIII were not toxic *in vivo*.

Although the actual mechanism by which its toxic action occurs cannot be commented on, it is possible that UpI may act by a phospholipase A-like action for the following reasons:

1. Its molecular weight of ~ 28 kDa, which is similar to the toxins (~ 30 kDa) produced by *Aiptasia pallida*,[1,2] the only sea anemone reported to have a phospholipase A_2-like activity.

2. Its partial amino acid sequence which has been shown to have some similarity to the bungarotoxins (Table 5.5) from the snake venom of *Bungarus multicintus* also known for their phospholipase activity.[40]

3. Its toxic action *in vitro* and *in vivo* as manifested by hemolysis, haemorrhage, edema and structural damage to erythrocytes of several mammalian species.

Based on the results presented in this thesis, it can be concluded that sea anemone extracts possess different biological activities. From the extract of *U. piscivora*, bioactive proteins have been isolated which clearly show different activities, in this case cardiotonic, hemolytic and cytotoxic. Three proteins: UpI, II and III were partially characterized. While two of the proteins (UpII and III) showed significant similarity to each other and appear to be isotoxins, UpI was clearly different. UpI was the most potent cardiotonic and hemolytic agent while UpII and III were devoid of any hemolytic activity. All three proteins were devoid of antifungal activity. A summary of the properties of the three proteins characterized is presented (Table 9.3), highlighting their significant similarities and differences.

In conclusion, although sea anemone toxins generally do not represent a public hazard, they deserve attention as toxins which may help us to understand a variety of membrane processes, as promising cardiac stimulatory compounds and potential sources of antitumor agents. Development of such drugs is an exciting but long term goal of

several laboratories investigating the sea anemone polypeptides. Besides the challenge of identifying receptor-binding domains on the polypeptide and designing simpler, possibly non-peptide analogs, it is necessary to minimize the predominant toxic effect of these substances.

The actual function of these polypeptides and proteins is a subject of controversy.[45] However there are suggestions that these toxins may be associated with chemical signaling for repelling predators.[5] Other possible functions include an acrorhagic response to ward off competition and ensure juvenile survivorship.[46] This is further strengthened by the fact that sea anemones are resistant to their own toxins.[5] The three proteins characterized here may function in one or all three categories, while at the same time possessing the biological activities that may have potential therapeutic implications. The fact that all three proteins possessed different activities may suggest different roles and functions within the anemone.

As further studies into the primary, secondary and tertiary structures of these toxins are investigated, and their biological activities evaluated, it is believed that these polypeptides and proteins will continue to serve as pharmacological and toxicological tools. They could also provide a means of chemotaxonomic or phylogenetic classification of sea anemones and become a future guide to Cnidarian toxinology.

Table 9.4 Comparison of characteristics of the cardiotonic proteins UpI, UpII and UpIII from *U. piscivora*

Characteristic	Proteins UpI	UpII	UpIII
Biological activity			
Cardiotonic	+	+	+
Hemolytic	+	-	-
Cytotoxic	+	nt	nt
Antifungal	-	-	-
Toxic (*in vivo* in rat)	+	-	-
Cardiostimulatory EC_{50} (M)	$8.1x10^{-9}$	$1x10^{-7}$	$7.6x10^{-6}$
Physicochemical			
Total protein (mg/ml)	0.1	0.1	0.3
Mol. wt. (kDa)[1]	27.2	18.7	18.6
Mol. wt. (kDa)[2]	28	19	19
Mol. wt. (kDa)[3]	28	40	40
pI	> 9.3	7.2	7.6
Partial sequence	+ (46)	+ (25)	+ (25)

(+) indicates positive activity or have been determined (-) indicates negative activity, Mol. wt. molecular weights (1) estimated from amino acid analysis, (2) estimated from SDS-PAGE in the presence and (3) absence of β-mercaptoethanol. Values in parenthesis indicates number of amino acid residues from N-terminal sequencing. nt (not tested)

9.2 Future considerations

Although three cardiotonic proteins were isolated from *Urticina piscivora*, further research needs to be carried out that will provide answers to some of the unanswered questions raised. One major area of study will be evaluating the primary, secondary and if possible tertiary structures of the toxins in order to be able to develop a cardiotonic or hemolytic structure activity relationship.

During the separation and purification procedures, only the cardiotonic proteins were considered. However, it would be interesting to determine if the proteins present in the other fractions possessed similar or other kinds of biological activities. Also elucidation of the primary structures of these other proteins would shed some light as to whether sea anemones possess polypeptides and proteins different from the three classes that are known today. This then would provide more information than can be used to propose legitimate arguments for reclassification of the cytolysins.

Further pharmacological evaluation of purified proteins would lend some understanding as to the mechanism of their cardiotonic action and their potential as therapeutic agents.

With regards to UpI, a complete primary structure would result in a better comparison to the bungarotoxins and allow speculation as to its mechanism of hemolytic action. The same can be said for UpII and III the isotoxins. Knowledge of the complete primary structure of UpII and UpIII will result in a better comparison of both and in

determining the extent of sequence homology. This could also lead to a cardiotonic structure activity relationship to determine if the active sequence is the same as for UpI.

Presently, the sea anemones are classified based on the structure of their tentacles.[47] Knowledge of which classes of polypeptide toxins reside within particular families could be a very useful basis for the chemotaxonomic re-classification of the sea anemones.

Bibliography

1. Budihna, M., Suput, D. and Macek, P. (1990) Cardiac arrhythmia and negative inotropic effect induced by equinatoxin II. *Int. Conf. IEEE Eng. Med. Biol. Soc.* **12**, 656-657.

2. Bunc, M., Frangez, R., Horvat, I., Turk, T., and Suput, D. (1994) Effects of equinatoxins *in vivo*. Possible role of degranulation of thrombocytes and granulocytes. *Toxins and Exocytosis. Ann. N. Y. Acad. Sc.* **710**, 162-167.

3. Shibata, S., Dunn, D. F., Kuchii, M., Kashiwagi, M. and Norton, T. R. (1974) Cardiac stimulant action of extracts of coelenterates on rat atria. *J. Pharm. Sci.* **63**, 1332-1333.

4. Kem, W. R. (1988) Sea anemone toxins: structure and action. In: *Biology of Nematocysts.* (Hessinger, D. A. and Lenhoff, H. M. eds.) pp. 375-405, Academic Press Inc. New York.

5. Macek, P. (1992) Polypeptide cytolytic toxins from sea anemones (Actinaria). *FEMS Microbiol. Immunol.* **105**, 121-130.

6. Turk, T. (1991) Cytolytic toxins from sea anemones. *J. Toxicol.-Toxin Rev.* **10**, 223-262.

7. Bernheimer, A. W. and Avigad, L. (1976) Properties of a toxin from the sea anemone *Stoichactis helianthus*, including specific binding to sphingomyelin. *Proc. Nat. Acad. Sci. USA* **73**, 467-471.

8. Bernheimer, A. W. and Avigad, L. (1978) A cholesterol-inhibitable cytolytic protein from the sea anemone *Metridium senile*. *Biochim. Biophys. Acta* **541**, 96-106.

9. Mahnir, V. M., Shumlov, U. N., Kovalevskaya, A. M., Romanenko, L. A. and Grebelny, S. D. (1993) Evidence for several types of biologically active substances in north Pacific sea anemones. *Comp. Biochem. Physiol.* **106C**, 661-665.

10. Mebs, D., Liebrich, M., Reul, A. and Samejima, Y. (1983) Hemolysins and proteinase inhibitors from sea anemones of the gulf of Aqaba. *Toxicon* **21**, 257-264.

11. Macek, P., Sencic, L. and Lebez, D. (1982) Isolation and partial characterization of three lethal and hemolytic toxins from the sea anemone *Actinia cari*. *Toxicon* **20**, 181-185.

12. Komatsu, K., Furukawa, K., Abe, K., Hirano, H. and Ueda, M. (1992) Isolation and characterization of equinatoxins from the sea anemone *Actinia equina* L. *Chem. Pharm. Bull.* **40**, 2873-2875.

13. Shiomi, K., Tanaka, E., Yamanaka, H. and Kikuchi, T. (1985) Isolation and characterization of a lethal hemolysin in the sea anemone *Parasicyonis actinostoloides*. *Toxicon* **23**, 865-874.

14. Bernheimer, A. W. and Lai, C. Y. (1985) Properties of a cytolytic toxin from the sea anemone, *Stoichactis kenti*. *Toxicon* **23**, 791-799.

15. Calton, G. J., Burnett, J. W. and Vader, W. (1977) A study of the nematocyst venom of the sea anemone *Bolecera tuediae*. *Toxicon* **16**, 443-451.

16. Norton, T. R., Kashiwagi, M. and Shibata, S. (1978) Anthopleurin A, B and C, cardiotonic polypeptides from the sea anemones *Anthopleura xanthogrammica* (Brandt) and *A. elegantissima* (Brandt). In: *Drugs and Food from the Sea.* (Kaul, P. N. and Sinderman, C. J. eds.) pp. 37-50, University of Oklahoma Press, Oklahoma.

17. Tanaka, M., Haniu, K., Yasunobu, K. T. and Norton, T. R. (1977) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant, anthopleurin A. *Biochem.* **16**, 204-208.

18. Shibata, S., Izumi, T., Seriguchi, D. G. and Norton, T. R. (1978) Further studies on the positive inotropic effect of the polypeptide anthopleurin-A from a sea anemone. *J. Pharmacol. Exp. Ther.* **205**, 683-692.

19. Reimer, N. S., Yasunobu, C. L., Yasunobu, T. and Norton, T. R. (1985) Amino acid sequence of the *Anthopleura xanthogrammica* heart stimulant anthopleurin-B. *J. Biol. Chem.* **260**, 8690-8695.

20. Thomson, M., Moritz, R. L., Simpson, R. J. and Norton, R. S. (1987) Tenebrosin-A, a new cardiostimulant protein from the Australian sea anemone *Actinia tenebrosa*. *Biochem. Int.* **15**, 711-718.

21. Khoo, K. S., Kam, W. K., Khoo, H. E., Gopalakrishnakone, P. and Chung, M. C. M. (1993) Purification and partial characterization of two cytolysins from a tropical sea anemone *Heteractis magnifica*. *Toxicon* **31**, 1567-1579.

22. Kem, W. R. and Dunn, B. M. (1988) Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon* **26**, 997-1008.

23. Hessinger, D. A., Lenhoff, H. M. and Kahan, L. B. (1973) Hemolytic, phospholipase A and nerve-affecting activities of sea anemone nematocyst venom. *Nature-New Biol.* **241**, 125-127.

24. Bernheimer, A. W. (1986) Proposed nomenclature for cytolytic toxins of sea anemones. *Toxicon* **24**, 1031-1032.

25. Alouf, J. E. and Geoffrey, C. (1984) Structure activity relationships in sulfhydryl activated toxins. In: *Bacterial Protein Toxins.* (Alouf, J. E., Fehrenbach, F. J., Freer, J. H., and Jeliaszewicz, J. eds.) pp. 165-175, Academic Press, London.

26. Bernheimer, A. W. and Avigad, L. S. (1982) Toxins of sea anemone *Epiactis prolifera*. *Arch. Biochem. Biophys.* **217**, 174-180.

27. Blumenthal, K. M. and Kem, W. R. (1983) Primary structure of *Stoichactis helianthus* cytolysin III. *J. Biol. Chem.* **258**, 5574-5581.

28. Simpson, R. J., Reid, G. E., Moritz, R. L., Morton, C. and Norton, R. S. (1990) Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and hemolytic protein from the sea anemone *Actinia tenebrosa*. *Eur. J. Biochem.* **190**, 319-328.

29. Spedding, M. and Berg, C. (1984) Interactions between a calcium channel agonist, Bay K 8644 and calcium antagonists differentiate calcium antagonist subgroups in K^+-depolarized smooth muscle. *Naun.-Schmeid. Arch. Pharmacol.* **328**, 69-75.

30. Scriabine, A., Van Arman, C. G., Morgan, G., Morris, A. A., Bennett, C. and Bohidar, N. R. (1979) Cardiotonic effects of anthopleurin A, a polypeptide from a sea anemone. *J. Cardiovas. Pharmacol.* **1**, 571-583.

31. Macek, P., Belmonte, G., Pederzolli, C. and Menestrina, G. (1994) Mechanism of action of equinatoxin II, a cytolysin from the sea anemone *Actinia equina* L. belonging to the family of actinoporins. *Toxicol.* **87**, 205-227.

32. Batista, U., Macek, P. and Sedmak, B. (1990) The cytotoxic and cytolytic activity of equinatoxin II from the sea anemone *Actinia equina*. *Cell Biol. Int. Rep.* **14**, 1013-1024.

33. Batista, U., Jezernik, K., Macek, P. and Sedmak, B. (1987) Morphological evidence of cytotoxic and cytolytic activity of equinatoxin II. *Period. Biolog.* **89**, 347-348.

34. Batista, U., Macek, P. and Sedmak, B. (1986) The influence of equinatoxin II on V-79-379 a cell line. *Period. Biolog.* **88**, 97-98.

35. Bernheimer, A. W., Avigad, L. S. and Kim, K. (1979) Comparison of metridiolysin from the sea anemone with thiol-activated cytolysins from bacteria. *Toxicon* **17**, 69-75.